



# THE JOURNAL OF ENDOCRINOLOGY

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# SERUM GONADOTROPHIN IN WELSH AND SHETLAND PONIES

By F. T. DAY AND I. W. ROWLANDS, *from the School of Agriculture, Cambridge University, and the National Institute for Medical Research, London, N.W. 3*

(Received 13 September 1945)

Cole [1938] observed that the concentration of gonadotrophin in the serum of mares during pregnancy was greater in small breeds than in large breeds; the maximum concentration in Welsh ponies was stated to be about four times that in draught breeds. In an earlier publication we [Day & Rowlands, 1940] recorded the time and rate of appearance of gonadotrophin in six Welsh ponies, a New Forest pony, and a Shetland pony. The maximum concentrations obtained were 43–186 i.u./ml. in the Welsh ponies and 84 and 344 i.u./ml. in the New Forest and Shetland ponies. No attempt was made to deal with individual or breed variation in the potency of the sera.

A considerable body of new information has accumulated in the past five years from two series of collections of serum gonadotrophin. One was made from a large number of Welsh ponies in Radnorshire and the other was made annually between 1940 and 1944 from each of a small number of Shetland ponies. A survey of the results is made below.

## MATERIAL AND METHOD

### *Animals*

Fifty *Welsh ponies* were bled in Radnorshire on 1–4 August 1940; thirty-five of these, which were 45–70 days pregnant, were bled again a fortnight later (13–16 August), together with twenty-five ponies which were bled for the first time. Pregnancy was diagnosed by rectal palpation of the uterus.

Six to nine *Shetland ponies* were bled annually in Cambridge, blood being withdrawn weekly for 6 weeks starting when the ponies were about 40 days pregnant. The ponies were kept in open pasturage in Cambridgeshire except during the mating season and early pregnancy. Ovulation was determined by palpation of the ovaries; pregnancy was produced by natural or artificial insemination and checked subsequently by palpation of the uterus.

### *Serum collection*

At each bleeding 2 l. of blood were withdrawn from the jugular vein into a sterile glass receptacle containing potassium oxalate. The serum, separated according to the method described by Rimington & Rowlands [1941], was Seitz-filtered and stored at  $-40^{\circ}\text{C}$ .

### *Assay of gonadotrophin*

The activity of the serum was assayed in immature female rats as previously described [Day & Rowlands, 1940] and expressed in international units.

GONADOTROPHIC POTENCY OF SERA FROM WELSH PONIES

The average potency of the serum collected in the total 110 bleedings was more than 100 i.u./ml. and over 150 i.u./ml. when the ponies were 60–80 days pregnant, which is the recognized period of maximum concentration of the hormone in the blood. The figures given in Table 1 show little evidence of the steep rise and fall in concentration

Table 1. *Comparison of potency and variability of sera of Welsh and Shetland ponies*

Stage of pregnancy (days)	40–49		50–59		60–69		70–79		80–89	
	No. of samples	Mean potency (i.u./ml. $\pm$ S.E.*)	No. of samples	Mean potency (i.u./ml. $\pm$ S.E.)	No. of samples	Mean potency (i.u./ml. $\pm$ S.E.)	No. of samples	Mean potency (i.u./ml. $\pm$ S.E.)	No. of samples	Mean potency (i.u./ml. $\pm$ S.E.)
Year										
(a) Welsh ponies										
1940	3	78 $\pm$ 30	7	130 $\pm$ 47	29	163 $\pm$ 27	45	141 $\pm$ 18	23	114 $\pm$ 14
(b) Shetland ponies										
1940	7	141 $\pm$ 27	11	273 $\pm$ 47	10	321 $\pm$ 28	9	270 $\pm$ 31	7	147 $\pm$ 30
1941	—	—	13	188 $\pm$ 22	11	251 $\pm$ 32	13	183 $\pm$ 24	13	85 $\pm$ 23
1942	10	127 $\pm$ 16	11	141 $\pm$ 14	13	214 $\pm$ 42	9	146 $\pm$ 54	8	111 $\pm$ 18
1943	16	48 $\pm$ 9	10	84 $\pm$ 18	13	88 $\pm$ 19	10	56 $\pm$ 18	8	73 $\pm$ 24
1944	14	65 $\pm$ 8	11	88 $\pm$ 17	14	75 $\pm$ 21	12	20 $\pm$ 5	9	19 $\pm$ 4

\* S.E.=standard error of the mean

shown in the previous small series of Welsh ponies [Day & Rowlands, 1940] which were bled in successive weeks. The discrepancy may be partly due to errors in estimates of the stage of pregnancy by the palpation method, but is probably mainly due to the great individual variations in the potency of the sera at similar stages of pregnancy.

As would be expected, the potency of the serum from the majority of animals first bled before the 60th day of pregnancy had increased at the second bleeding 14 days later. Even when the first bleeding was made when the ponies were 70–79 days pregnant, five of twelve showed a greater potency a fortnight later. These figures given in Table 2 illustrate the individual variation in the time of maximum concentration of the hormone which may occur any time within the period of the 45th–79th days of pregnancy.

Table 2. *Comparison of average potency of serum of Welsh ponies bled twice*

Stage of pregnancy (days)	No. of ponies	Potency of sera (i.u./ml.)	Potency of sera 14 days later (i.u./ml.)	% of ponies having serum of increased potency at 2nd bleeding
45–49	3	116	183	100
50–59	4	151	148	75
60–69	15	155	170	47
70–79	12	153	128	40
80–89	1	42	15	0

GONADOTROPHIC POTENCY IN SERA FROM SHETLAND PONIES

In all thirteen ponies were studied during 1940–4, and most of them during 2–5 successive pregnancies during that time. The average concentrations of serum gonadotrophin at different stages of pregnancy are recorded in Table 1, and in Table 3 are

Table 3. *Annual yield of serum gonadotrophin from Shetland ponies*

Year	No. of ponies pregnant	No. of bleedings	Total volume of serum (l.)	Total activity (i.u. $\times 10^6$ )	Mean potency (i.u./ml.)
1940*	75	112	106.00	14.080	133
1940	8	39	48.08	12.408	258
1941	9	54	70.14	12.162	173
1942	6	51	62.50	9.563	153
1943	7	50	75.00	5.473	73
1944	9	64	80.00	3.371	42

\* Data of serum collected from Welsh ponies included for comparison

given the average concentrations in the total amounts of serum collected in the different years. From these two tables it is evident that in 1940 the gonadotrophic potency in the Shetland ponies was about double that in the Welsh ponies.

#### *Potency in successive pregnancies*

The relatively high potency attained in 1940 was not, however, maintained and each succeeding year saw a fall in the average maxima attained and in the average potency of the total collection (Tables 1 and 3).

At present no satisfactory explanation can be offered of this disturbance in endocrine secretion. The average potency of the sera in the three ponies (L, M and N) that carried foals in each of the 5 years decreased from an average of 245 i.u./ml. in 1940 to one of 63 i.u./ml. in 1944; but a similar decline took place in those ponies that had 2-year intervals between successive pregnancies or which were only bled twice before the final bleeding in 1944 (J, T, U and W)—these results are graphed in Fig. 1, which shows that the individual trends in all the ten ponies which had three or more pregnancies were very similar. The fall does not appear to have been related to age or parity, nor is it likely to have been the result of some cumulative effect of the withdrawal of blood. It may be caused by changes in the environment. Although the disturbance did not appear to have been detrimental to pregnancy it seriously interfered with the collection of gonadotrophin, the total quantity obtained in 1944 being only about a quarter of that obtained in 1940 (Table 3).

#### *Effects of sex hormones*

*Oestrogen.* Cole & Saunders [1935] drew attention to the fact that the decrease in the concentration of gonadotrophin in the serum coincides with a rise in the urinary excretion of oestrogens in the pregnant mare. In view, therefore, of the well-known inhibitory action of large doses of oestrogen on the secretion of pituitary gonadotrophin, it seemed possible that the decreased gonadotrophic potency of the serum might be connected with an increase of oestrogen even if the gonadotrophin of mare serum has its origin in some other organ than the pituitary gland. An experiment was therefore carried out in which the oestrogen level in five ponies during early pregnancy was artificially raised by implanting them subcutaneously with stilboestrol tablets.

The total amount of stilboestrol implanted as 50 mg. tablets was 47–503 mg. This treatment had no effect on serum gonadotrophin concentration but caused abortion in three of the ponies within 4–7 weeks. The concentration of gonadotrophin was higher in the aborting ponies than in those that delivered successfully. The least

amount of stilboestrol absorbed by any pony to cause abortion was calculated to be about 10 mg. (mare X, 1943) which amount in proportion to body weight is considerably less than that (0.02 mg.) necessary to cause foetal resorption in the rat [Hain, 1937].

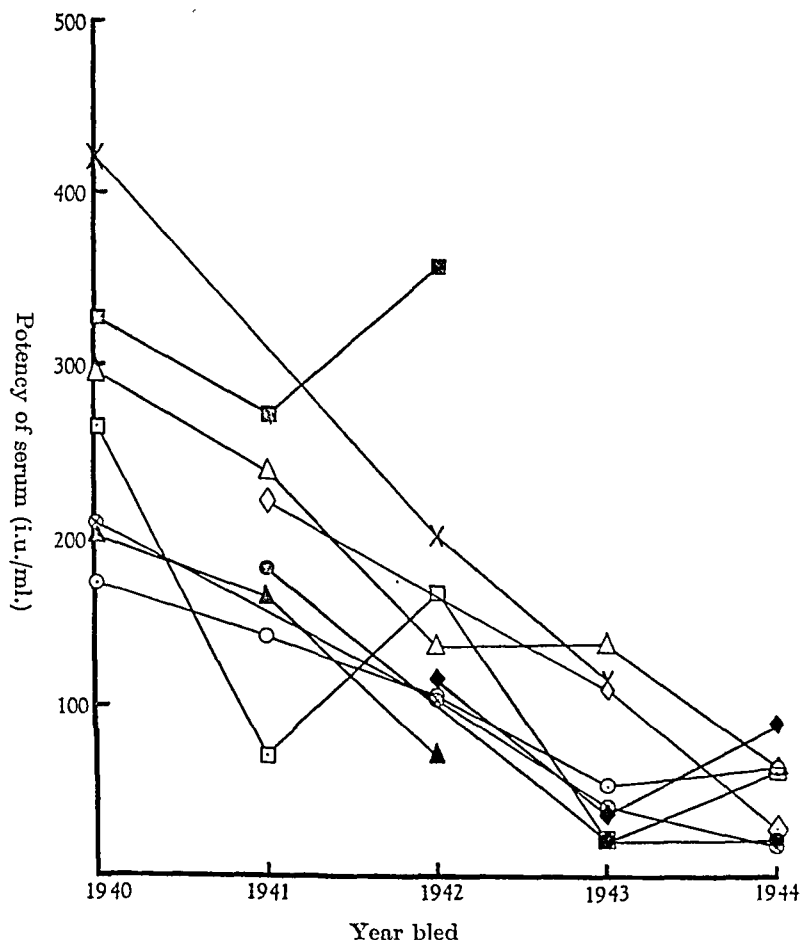


FIG. 1. Average potency of serum of Shetland ponies in successive pregnancies. Only ponies which were bled in 3 or more years are included; ages in 1940 and hormone treatments are given in parentheses. × mare J (13; stilboestrol 1943). ○ mare L (13; testosterone 1942). △ mare M (12; stilboestrol 1942; testosterone 1943). □ mare N (6; nil). ■ mare P (3; nil). ▲ mare Q (7; stilboestrol 1942). ⊗ mare R (6; stilboestrol 1943). ◇ mare T (3; ethisterone 1943). ◆ mare U (2; nil). ● mare W (2; nil).

Details of treatment and results are given below (see also Fig. 1).

*Mare M* (1942). Implanted ten tablets, weight 503 mg., on 38th day. Pregnancy normal to 57th day. Aborted on 90th day. Foetal development 70–75 days. Tablets removed on 87th day, weight 412 mg. Amount absorbed in 49 days: 91 mg. or 10.3% in 1 month. Potency of serum (i.u./ml.) on 45th day and subsequently at weekly intervals: 105, 136, 140, 170, 100, 156 and 128. Abortion occurred 3 days after last bleeding.

*Mare Q* (1942). Implanted five tablets, weight 235 mg., on 41st day. Pregnancy normal on 66th day and again on 71st day when pony killed by enemy action. Tablets removed on 71st day; only four recovered, weight 147 mg. Amount absorbed from original calculated weight of four tablets (188 mg.) in 30 days: 41 mg. or 22% in 1 month. Potency of serum (i.u./ml.) on 43rd day and subsequently at weekly intervals: 30, 70, 80, 80 and 85.

*Mare J* (1943). Implanted five tablets, weight 229.5 mg., on 34th day. Pregnancy normal to 54th day. Aborted 60th day. Tablets removed on 63rd day, weight 198 mg. Amount

absorbed in 1 month: 31.5 mg. or 13.8%. Potency of serum (i.u./ml.) on 40th day and subsequently at weekly intervals: 76, 135, 107, 170 and 92. Abortion occurred 1 day before bleeding.

*Mare R* (1943). Implanted two tablets, weight 90.2 mg., on 31st day. Pregnancy normal to 84th day. Tablets removed on 106th day, weight 46.1 mg. Amount absorbed in 10 weeks: 44.1 mg. or 19.6% in 1 month. Gave birth to normal colt foal. Potency of serum (i.u./ml.) on 37th day and subsequently at weekly intervals: 26, 40, 37, 46, 75, 48, 20, 10, 7, 15 and 12.

*Mare X* (1943). Implanted one tablet, weight 47.0 mg., on 26th day. Pregnancy normal to 72nd day, but afterwards foetus failed to develop; aborted between 81st and 88th day. Tablet after 8 weeks' implantation could not be found. Potency of serum (i.u./ml.) on 40th day and subsequently at weekly intervals: 6, 28, 122, 210, 220, 185, 210, 80, 100 and 32. Abortion occurred between 7th and 8th bleeding.

*Androgen.* Androgens inhibit the capacity of oestrogens to cause vaginal cornification [Robson, 1936], and it seemed possible, therefore, that any effect of oestrogens on the production of gonadotrophin in the serum of the pregnant mare might be antagonized by the maintenance of a high concentration of androgen in the blood.

Three ponies were therefore implanted with 40-100 50 mg. tablets of testosterone during early pregnancy. The concentrations of gonadotrophin in mares L and N in 1942 were not abnormal for that year though the value in the last was higher than in the same pony in the preceding or following year. Mare M, treated in 1943, showed the highest average concentration of any pony in that year except mare X, which was implanted with stilboestrol. It is doubtful whether these apparent rises are significant.

All three ponies carried their foals to term and were delivered at the expected date. Hain [1937] and Sciapedes [1937] observed foetal absorption in rats injected with testosterone. The amount required to produce this effect in the rat was, however, considerably greater in proportion to body weight than that absorbed by the pony implanted with 5 g. of testosterone.

Details of treatment and results follow (see also Fig. 1).

*Mare L* (1942). Implanted forty tablets, weight 1.982 g., on 56th day. Pregnancy continued normally. Tablets could not be palpated on 127th day and no trace of them was found. Assumed that absorption was complete. Potency of serum (i.u./ml.) on 41st day and subsequently at weekly intervals: 95, 180, 110, 90, 44, 50 and 68. Gave birth to a normal filly foal at expected date.

*Mare N* (1942). Implanted forty tablets, weight 1.970 g., on 60th day. Pregnancy normal when examined on 97th day and tablets could be palpated. On 111th day, pocket containing tablets had been broken and only 2½ badly chipped tablets could be found in the surrounding hair. Potency of serum (i.u./ml.) on 48th day and subsequently at weekly intervals: 200, 190, 300, 130, 50 and 115. Gave birth to normal colt foal at expected date.

*Mare M* (1943). Implanted 100 tablets, weight 4.951 g., on 27th day. By 54th day a slight stitch suppuration had developed, no tablets extruded, but tablet clump was small suggesting disintegration. Pregnancy normal on 94th day. Only one fragile tablet was recovered, and this crushed on handling; the remainder were probably completely absorbed. Potency of serum (i.u./ml.) on 39th day and subsequently at weekly intervals: 51, 75, 190, 210, 140, 126, 80 and 54. Gave birth to normal filly foal at expected date.

No evidence is available of the amount of testosterone absorbed in any one of these ponies. After 10 weeks' implantation, the tablets in mares L and M were assumed at the time of examination to have been completely absorbed. Such an assumption in view of Emmens' [1941] results in rats, showing that free testosterone from a tablet weighing 40 mg. is absorbed at the rate of about 38% per month, is probably correct.

*Progestogen.* One pony (mare T) was injected in 1943 with 5 g. of ethisterone. This had no effect on the output of serum gonadotrophin. It may be remarked, however, that



as this pony aborted spontaneously in 1941 and 1942 the injection of this progestogen might very well have contributed to the success of the pregnancy in 1943.

*Mare T* (1943). Injected subcutaneously with a suspension of 5 g. of ethisterone in 20 ml. saline on 22nd day. Pregnancy normal at 96th day and suspension could be palpated. Potency of serum (i.u./ml.) on 41st day and subsequently at weekly intervals: 38, 120, 150, 200, 55, 48, 11 and 15.

## DISCUSSION

### *Methods of serum collection*

The results have shown that ponies of both breeds are good donors of serum gonadotrophin, but that disadvantages are attached to the methods of collection described above. The first method of single bleedings is inefficient because of the very great

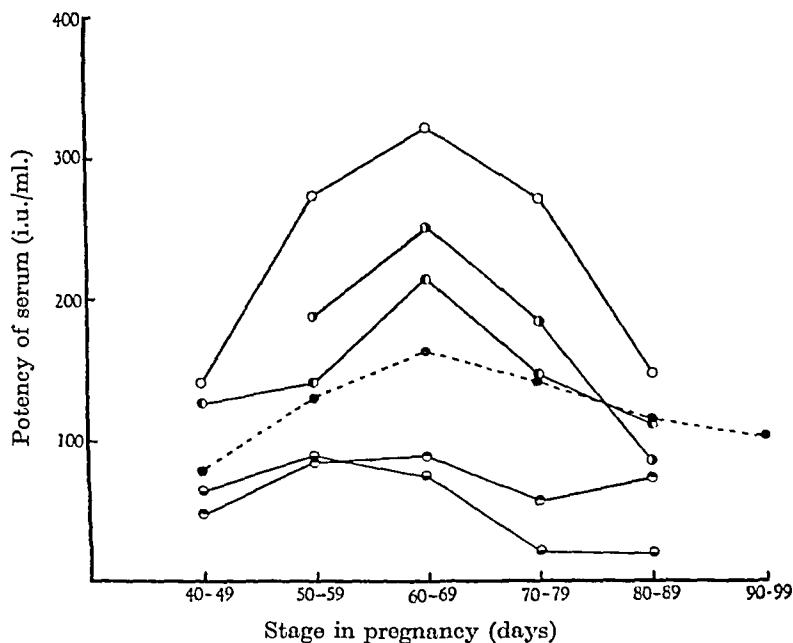


FIG. 2. Average potency of sera of Welsh and Shetland ponies in different stages of pregnancy 1940-4. ○ Shetland ponies in 1940. ● Shetland ponies in 1941. ○ Shetland ponies in 1942. ● Shetland ponies in 1943. ○ Shetland ponies in 1944. ● Welsh ponies in 1940.

individual variation in the hormone content of the serum of Welsh ponies at similar stages of pregnancy. For instance, it can be calculated that one-half the total unitage of hormone collected from these ponies was contributed by serum containing 200 or more i.u./ml. which represents but one-fifth of the total number of ponies bled. The discovery of a criterion by which ponies secreting high and low quantities of gonadotrophin could be rapidly distinguished in the field would do much to facilitate a more economical collection and provide a pool of serum having a greater mean potency. The second method of repeated bleedings of Shetland ponies in successive pregnancies leads to an annual decrease in the amount of hormone secreted.

### *Differences between the two breeds*

The time of appearance and of the attainment of maximal concentration of the hormone in the serum appeared to be the same in both breeds, although at all stages examined during the 1940 breeding season the amount present in the Shetland ponies

was about twice that in the somewhat larger Welsh ponies (Table 1). It will be noted, too (Fig. 2), that the curve of hormone concentration in the serum of the Shetland ponies in the same year rises and falls more steeply than does that in the Welsh ponies. This peak, however, disappeared completely by the 4th year (1943) of collection. The difference in the shape of the 1940 curves for the two breeds is perhaps accentuated by the more extensive data obtained for the Welsh ponies. Individual variation in potency was about the same in the two breeds at all stages of pregnancy and in all the years when serum was collected.

*Factors affecting the secretion of hormone*

A search has been made, so far unsuccessfully, for the factor(s) which determine the concentration of gonadotrophin in the serum of the pregnant pony, with a view to controlling the variability in the potency of the individual sera. Some of these are considered below.

*Age of pony.* The age of all the Welsh ponies was recorded by inspection of the teeth; they were found to vary between 2 and 10 or more years. In view of the high rate of fertility observed in 1940 it is probable that the number of foals previously borne by these ponies varied from none to eight or more. It can be seen from Table 4 that, with

Table 4. *Lack of correlation between age of pony and potency of serum*

Stage of pregnancy (days)	Mean potency of serum (i.u./ml.) in ponies aged (years)								
	2	3	4	5	6	7	8	9	10+
60	35 (2)*	53 (3)	210 (5)	180 (4)	83 (3)	140 (1)	—	260 (4)	—
70	—	110 (9)	94 (5)	158 (4)	130 (1)	49 (3)	287 (3)	148 (2)	150 (2)
80	70 (1)	128 (2)	70 (4)	75 (1)	—	—	—	280 (1)	68 (2)

\* No. of ponies in parentheses.

the possible exception of the 2-year ponies, presumably carrying their first foals, no relation exists between age and potency of serum. A similar lack of correlation has already been noticed in the Shetland ponies (see Fig. 1).

*Diet.* The decrease in the potency of the sera of the Shetland ponies in 1943 was thought to be due to inadequate food supply at the time of bleeding. The physical condition of the ponies was poor; two of them (mares N and U) aborted spontaneously. In the following year (1944) precautions were taken to ensure that the ponies received an adequate diet, and during early pregnancy they were maintained in good condition by a liberal supply of high-quality sainfoin hay. In spite of this, the individual variation in the output of gonadotrophin by the ponies remained high, and the mean potency of the serum showed a further decrease. No spontaneous abortions occurred. We agree with Cole [1938], therefore, that no relation exists between the potency of the serum and the nutritional state of the animal.

*Sex hormones.* The experiments concerned with the effect of sex hormones in pregnancy in the mare have been given in some detail on account of their interest in other connexions. As for their effect on gonadotrophin concentration, however, the results were essentially negative.

*Other factors.* Other possible causes for the variability in the potency of individual sera connected with the methods employed in their collection may be enumerated, such as (a) inaccuracies in the estimation of the stage in pregnancy, (b) differential

thermostability during the collection period, and (c) errors involved in biological assay. None of these factors satisfactorily explains the very large differences obtained in the potency of the sera at similar stages of pregnancy.

#### SUMMARY

1. Observations have been made on the gonadotrophic potency of the sera from seventy-five Welsh ponies bled in Wales in 1940, and from a small number of Shetland ponies kept in Cambridge during successive pregnancies between 1940 and 1944.

2. The average potency of the serum from the Welsh ponies between the 45th and 100th day of pregnancy was 133 i.u./ml., while that from the eight Shetland ponies, pregnant in 1940, was 258 i.u./ml.

3. The concentration of the hormone in the Shetland ponies decreased in succeeding pregnancies, so that by 1944 the mean potency of the serum of nine ponies was only about 40 i.u./ml.

4. The variability in the potency of the individual sera from both breeds is high, and in the Shetlands it was proportional to the mean potency in all years of collection.

5. The individual variability and the diminishing rate of secretion in succeeding pregnancies did not appear to be related to (a) age or parity of the animal, (b) diet, or (c) concentration of gonadal hormones in the blood.

6. Stilboestrol exerts a powerful abortifacient action in the mare.

The collection of serum from the Welsh ponies in 1940 was supported financially by the Agricultural Research Council, to whom we express our thanks for permitting us to retain a sample of each serum for biological assay. We also record our appreciation of the invaluable assistance given by Messrs E. F. Hughes and S. J. Tadman of the Field Laboratories of the Institute of Animal Pathology, Cambridge, in the collection of blood and preparation of serum. We are indebted to Dr Franklin Kidd, F.R.S., for the allocation of storage space in the Low Temperature Station at Cambridge and to Mr Smart, of 'Ciba' Ltd., for a gift of testosterone.

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# THE MEASUREMENT OF LACTATIONAL PERFORMANCE IN THE RAT IN STUDIES OF THE ENDOCRINE CONTROL OF LACTATION

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A serious difficulty in lactational studies on small laboratory animals is the lack of a satisfactory means of milking them and thus directly measuring their milk yields. At present, lactational performance in rats can only be inferred from litter growth and survival data determined under carefully standardized conditions. Comparison of litter-growth curves (and survival data) for different groups of rats gives some idea of their relative lactational performances but it is not possible to calculate daily milk yields from such curves since the weight losses due to the excreta and insensible perspiration are unknown.

Attempts to determine true milk yields by the complicated and not entirely unobjectionable (see discussion by Enzmann) procedures used by Enzmann [1933] for mice and by Brody & Nisbet [1938] for rats, which involve determination not only of the daily litter weight gains but also of the daily losses due to the excreta and perspiration, are clearly impracticable in experiments carried out on any considerable scale. Thus when, in the course of studies of the relation between the adrenal cortex and lactation [Cowie & Folley, 1946*a*, *b*], we wished to determine quantitatively the lactational responses of adrenalectomized rats to treatment with adrenal cortex hormones, we had to devise a quantitative criterion of lactation which was derived solely from the ordinary litter-growth curve.

## THE EQUATION OF THE LITTER-GROWTH CURVE

Daggs [1935] proposed a 'lactation index' for rats based on the claim (in which respect he followed Brody [1927]) that the points obtained by plotting the logarithm of the mean weight of the young against time fall along two straight lines which intersect at approximately  $t=10$  days. The numerical index used by Daggs has, however, little theoretical significance and cannot be used for quantitative comparisons even if the premises on which it is based are true.

We have constructed a mean growth curve (Fig. 1, circles) for the combined litters of a group of twenty-seven sham-operated rats, composed of control groups used in studies of the role of the adrenal cortex in lactation [Folley & Cowie, 1944; Cowie & Folley, 1946*a*, *b*]. Particulars of the diet and general experimental procedure are given elsewhere [Cowie & Folley, 1946*a*, *b*]. Analysis of the data used for the construction of our curve failed to confirm Daggs' claim, at any rate as applied to our conditions, since the points obtained by logarithmic plotting, as specified above, fall on a smooth curve to which a 'forced' fit of two intersecting straight lines could be made, but which could only be regarded as an approximation with no theoretical

foundation. In fact, inspection of this growth curve (Fig. 1), which may be regarded as representative of litter-growth data for our rats under the conditions defined, shows that over the period during which the young are solely dependent on their mothers' milk, i.e. from birth to 16 days, the curve is sigmoid in shape and we have shown (Fig. 1)

that it can be closely fitted over the whole range by the logistic:  $w = \frac{40.42}{1 + e^{(9.6-t)/5.09}}$ ,

where  $w$  = the mean weight of the young at time  $t$ . This equation is of the same form as that derived by Robertson [1907-8] on theoretical grounds to express individual growth rates.

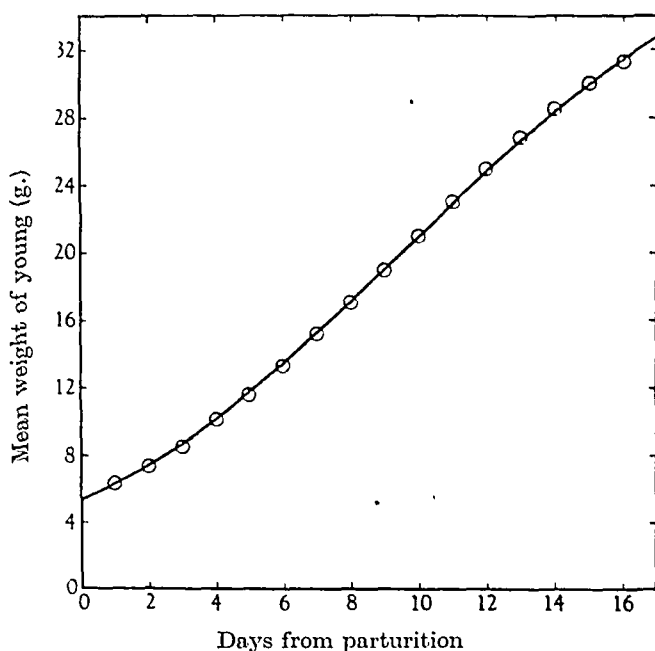


FIG. 1. Observed values (circles) for the mean weight of the young of twenty-seven sham-operated rats having a total of 216 pups on the 1st day of lactation and the logistic curve (unbroken line) which gives a good fit to the points. The equation of the logistic curve is  $w = \frac{40.42}{1 + e^{(9.6-t)/5.09}}$ , where  $w$  = the mean weight of the young at time  $t$ .

In Table 1 are given in addition to the observed growth data for the group of litters in question, values calculated from the above logistic together with values calculated from logarithmic equations, fitted by the method of least squares, to the observed values for the two intervals from the 4th-10th and the 10th-16th days respectively. This is the procedure used by Daggs except that he extended the second interval to the 17th day and fitted his straight lines by eye. It is evident that the logistic gives a better fit over the whole range than either of the logarithmic equations over its restricted range. The point of inflexion of the logistic comes at  $t = 9.6$  days, which is close to the values given by Brody [1927] and Daggs [1935] as the point of intersection of the logarithmic curves fitting their data, but it is clear from what has been said that our litters experienced no such sharp discontinuity in growth at this time as postulated by these authors.

This last point is important in the derivation of a quantitative measure of lactational performance since it is necessary to replace Daggs' approximation with another which

Table 1. *Comparison of observed and calculated values for the mean weights of the litters of twenty-seven sham-operated rats with a total of 216 pups on the first day of lactation*

Days from parturition (t)	Mean wt. of young (obs.) g.	Mean wt. (w) of young calculated from equation		
		$w = \frac{40.42}{1 + e^{(9.6-t)/5.09}}$ g.	$\log_{10} w = 0.051t + 0.82$ g.	$\log_{10} w = 0.027t + 1.07$ g.
1	6.36	6.30	—	—
2	7.32	7.42	—	—
3	8.54	8.61	—	—
4	10.08	10.13	10.55	—
5	11.59	11.66	11.85	—
6	13.31	13.35	13.31	—
7	15.20	15.16	14.96	—
8	17.05	17.06	16.80	—
9	19.03	19.01	18.87	—
10	21.02	21.00	21.19	21.78
11	23.02	22.97	—	23.16
12	24.96	24.86	—	24.63
13	26.83	26.62	—	26.20
14	28.48	28.41	—	27.87
15	30.04	29.98	—	29.65
16	31.34	31.45	—	31.54

we believe is better founded, but, if only because of the labour involved in fitting a logistic curve to a given set of data, use of this expression, well as it fits the facts, is hardly practicable for the present purpose.

#### MEASUREMENT OF LACTATIONAL PERFORMANCE

As there is no discontinuity in the growth curve at the 9th–10th day, the points corresponding to the 5-day period from the 6th to the 11th days of lactation lie very close indeed to a straight line, so that for a group of rats over this period the mean daily increase in total litter weight per rat is approximately constant and, incidentally, at a maximum. This quantity, which we shall use as a quantitative measure of the efficiency of lactation, we propose, to avoid confusion with the ‘lactation index’ of Daggs, to call the ‘litter-growth index’. The period chosen has the further advantage that in the case of rats undergoing operation at the 4th day, as in our experiments involving adrenalectomy [Cowie & Folley, 1946*a*, *b*], 2 days are allowed for recovery from the effects of the operation and for the stabilization of any effects on lactation of the operation and of any replacement therapy instituted at the same time.

It must be understood that the litter-growth index can only be regarded as a first approximation to being proportional to the true milk yield, since the weight losses of the young attributable to the excreta, insensible perspiration and other causes, which are neglected, may not be simply related to the true milk yield. However, analysis of the data of Brody & Nisbet [1938] indicates that the litter-growth index is a fairly constant percentage (roughly 50–60 %) of the true milk yield. Its validity as a quantitative measure of lactation also rests on the assumption that there are sufficient young in the litter to use all the milk secreted by the mother. This we believe to be the case in our experiments since it is our practice to work with litters of eight, equally distributed between the sexes wherever possible (though owing to deaths among the young and the practical necessity of including a few rats with litters of

seven, the average litter size by the 6th day is usually slightly less than this) and with litters of this size the growth rate increases when extra food becomes available soon after the 16th day. For comparative purposes it is obviously necessary that the mean litter size shall be kept as constant as possible from one experiment to another and further, data for any litter which has suffered reduction during the 5-day period should be omitted from the calculation, since the latter involves the total litter weight, not the mean weight per pup.

#### MEASUREMENT OF PARTIAL LACTATIONAL INHIBITION

This method of assessing lactational performance is applicable to rats which have been subjected to procedures, such as adrenalectomy, which cause a partial depression of lactation [Folley & Cowie, 1944; Cowie & Folley, 1946*a*, *b*], provided that the depressed growth curves are reasonably linear between the 6th and 11th days and that no deaths among the litters occur during this period. It is inapplicable in extreme cases where lactation is so severely depressed that the growth curves are deflected downwards very soon after the operation. In such cases a litter-growth index of zero can be assumed without much error. In the more usual event of partial inhibition, where enough milk is secreted to carry all the young for a time but at a reduced rate of growth, a convenient inverse measure of the degree of lactational inhibition is given by the litter-growth index for the operated rats expressed as a percentage of that for simultaneously run controls.

#### LACTATIONAL RESPONSES TO REPLACEMENT THERAPY

In devising a quantitative measure of the lactational responses to replacement therapy in rats which have been subjected to adrenalectomy or other endocrine operations which interfere with lactation, the question arises whether the absolute intensity of lactation attained should be taken as the response or whether the lactational performance of simultaneously run sham-operated and operated but untreated control groups should be taken into account. Since litter-growth indices for such groups of our rats vary to some extent from one experiment to another [Cowie & Folley, 1946*a*] there seems little doubt that the second alternative should be followed. Accordingly we have adopted as a measure of such responses the increase in the litter-growth index due to the treatment expressed as a percentage of the theoretical maximum. Thus if  $a$ ,  $b$ , and  $c$  are the indices for control, operated-untreated, and operated-treated groups respectively, the response is given by  $\frac{100 (c - b)}{a - b}$ .

#### SUMMARY

1. The mean growth curve of the litters of a group of lactating rats was closely fitted, over the whole period during which the young are solely dependent on their mothers' milk, by a logistic equation. Previous claims of the existence of a sharp discontinuity in the growth rate at about the 10th day have not been confirmed.

2. The 'litter-growth index' for a group of rats is defined as the mean daily gain in weight per litter over the 5-day period from the 6th to the 11th days. The use of this quantity as an approximate measure of lactational performance enables the effects on lactation of endocrine factors to be studied quantitatively.

We are greatly indebted to Dr S. K. Kon for placing at our disposal the facilities of the rat colony maintained by him at this Institute for nutritional investigations and to Dr A. C. Bottomley for advice regarding the fitting of the logistic curve. The help of the Agricultural Research Council who provided one of us (A.T.C.) with a special research grant is gratefully acknowledged.

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# ADRENALECTOMY AND REPLACEMENT THERAPY IN LACTATING RATS

## 2. EFFECTS OF DEOXYCORTICOSTERONE ACETATE ON LACTATION IN ADRENALECTOMIZED RATS

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It is now well established that the numerous physiological disturbances resulting from adrenalectomy include a marked inhibition of lactation (see reviews by Turner [1939], Folley [1940] and Petersen [1944]). In a previous study of replacement therapy in adrenalectomized lactating rats [Folley & Cowie, 1944] we have shown that in our rats and under the conditions of our experiments, deoxycorticosterone acetate was more effective in maintaining lactation than either 11-dehydrocorticosterone or 17-hydroxy-11-dehydrocorticosterone. Neither with the steroid nor an extract of adrenal cortex, however, did we find it possible, with the doses given, to achieve complete restoration of lactation as judged by the only available criterion, the growth rates of litters of standard size measured under strictly controlled conditions. Our results differed from those of Gaunt, Eversole & Kendall [1942] who obtained normal lactation in adrenalectomized rats treated with 17-hydroxy-11-dehydrocorticosterone or with an extract of adrenal cortex, but only partial and inconsistent maintenance with deoxycorticosterone acetate.

In our experiments two doses of deoxycorticosterone acetate were used and a graded dose-response relation was obtained. It thus seemed likely that still greater doses would give better, perhaps complete, maintenance of lactation. We have therefore investigated the dose-response relations over a wider range of dosage. It seemed important to confirm if possible the existence of a regular relation between dose and response as Gaunt *et al.* [1942] reported erratic results with deoxycorticosterone acetate and Gaunt [1941] had earlier found that this steroid brought about no improvement of lactation in adrenalectomized rats. The existence of such a regular dose-response relation would provide additional evidence that the favourable effect of deoxycorticosterone on lactation in our adrenalectomized rats is a real and reproducible phenomenon.

We have also examined the degree of inhibition of lactation produced by adrenalectomy and its relation to the lactational performance of simultaneously studied control rats.

### EXPERIMENTAL

#### *Methods*

In all save one experiment (see p. 22), the rats used were uniparous females  $4\frac{1}{2}$ –5 months old; their diet and the general experimental procedure were as described previously [Folley & Cowie, 1944]. The stock diet, which was fed *ad lib.*, has been found to contain 0.40% Na<sub>2</sub>O and 0.95% K<sub>2</sub>O calculated on a moisture-free basis. In addition the

rats received cows' milk *ad lib.*, raw liver twice weekly (approx. 5 g./rat) and raw carrot daily except Sundays; the effect of these supplements on the intake of Na and K is not likely to be great [see Cowie & Folley, 1946a].

As before, adrenalectomy was performed on the 4th day of lactation and all control rats were subjected to a sham operation. Deoxycorticosterone acetate was subcutaneously injected once daily, in most experiments from the day of operation until the day before weaning (see Table 3). The hormone was dissolved in sesame oil in such concentration that the daily dose was usually contained in 0.5 ml. In all save a few instances untreated rats received similar injections of sesame oil alone. No differences in lactational performance have been found between untreated and oil-injected controls [Folley & Cowie, 1944].

#### *Effect of adrenalectomy on lactation*

We had previously found [Folley & Cowie, 1944] that in our rats the effects of adrenalectomy on lactation were less severe than those observed by Gaunt *et al.* [1942]. We have now acquired sufficient data to present a more generalized picture of effects of

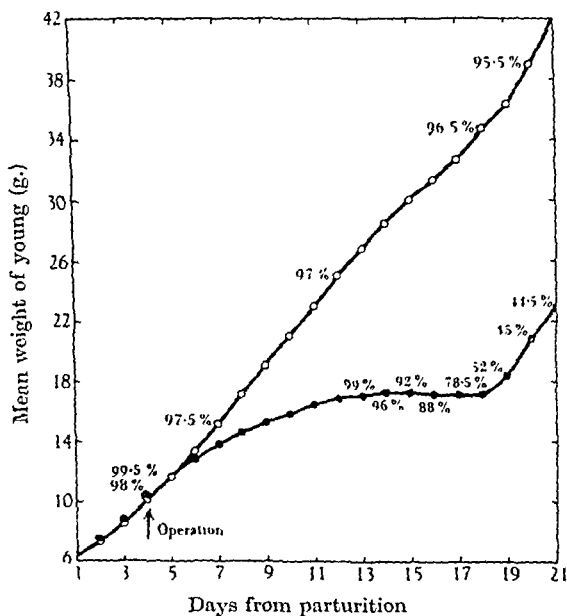


FIG. 1. Mean growth curves of litters of composite groups of adrenalectomized and sham-operated rats. O—O, litters of 27 sham-operated rats with a total of 212 pups on the 4th day of lactation. ●—●, litters of 27 adrenalectomized rats with a total of 204 pups on the 4th day of lactation. The figures near the curves give the percentages of pups surviving.

adrenalectomy on lactation in rats of this colony. The mean growth curves of the litters of composite groups of adrenalectomized and sham-operated rats made up of groups used in several separate experiments are shown in Fig. 1. These curves, which may be taken as generally representative for our rats, show, in confirmation of our previous findings, that under the conditions of our experiments, adrenalectomy does not in general cause complete inhibition of lactation. In this colony the 16th day of lactation marks the end of the period over which the growth curve of the litter may

be taken as a criterion of the lactational performance of the mother, since the eyes of the young open at about this time and thereafter they have access to the food provided for the mother. From Fig. 1 it will be seen that at the 16th day 88 % of the young of the adrenalectomized rats were still alive as compared with a 97 % survival of the control young which, however, were almost twice as heavy. At weaning the respective survival rates for the young of adrenalectomized and sham-operated rats were respectively 44.5% and 95.5 % and the mean weight per pup of the former was approximately 50 % of that of the latter. These results denote a marked, but far from complete, inhibition of lactation. That this is not invariably the case will, however, be seen later.

The possibility arises that the partial nature of this inhibitory effect is due to the presence in our adrenalectomized rats of sufficient accessory cortical tissue to allow lactation at a reduced level. If so it might be possible to improve the lactational performance of such rats by administration of anterior-pituitary adrenotrophic hormone which should produce functional hypertrophy of any accessory cortical tissue. The results of an experiment in which 1 unit daily of a purified preparation of adrenotrophin containing at least 32 sudanophobic units [Reiss, Bálint, Oestreicher & Aronson, 1936] per mg. was administered to adrenalectomized rats are given in Table 1. It will be seen that lactation was not improved by the administration of this preparation, indeed the lactational performance of both intact and adrenalectomized rats receiving the hormone was slightly inferior to that of the relevant controls.

Table 1. *Effect of treatment with adrenotrophin and deoxycorticosterone acetate (doca) on lactation in adrenalectomized rats*

On lactation in adrenalectomized rats												Per cent change in wt. of mothers between 4th and 21st days
Treatment	No. of mothers	Total no. of young on 4th day		Mean wt. (g.) of young on day			Per cent of young alive on day			No. of mothers dead by 21st day	No. of litters dead by 21st day	
		♂	♀	4	16	21	4	16	21			
Sham-operated rats												
No treatment	6	25	20	10.3	32.2	43.1	94	94	94	0	0	+ 13.6
Adrenotrophin*	6	30	18	10.4	30.9	41.6	100	100	100	0	0	+ 7.6
Adrenalectomized rats												
No treatment	6	24	20	10.2	17.0	22.5	98	98	22	0	4	- 8.5
Adrenotrophin*	7	36	18	9.8	15.8	19.4	98	69	25	2	4	- 10.8†
doca: 0.1 mg.‡	6	23	21	10.1	20.5	23.4	94	72	62	0	1	+ 5.5
doca: 1.0 mg.‡	5	17	19	9.9	23.2	33.2	100	100	81	0	1	+ 18.4
doca: 3.0 mg.‡	6	24	21	10.6	25.9	34.8	100	100	100	0	0	+ 16.9

\* Each rat received 17 daily subcutaneous injections of 1 Sudanophobic unit [Reiss *et al.* 1936] of a preparation of purified adrenotrophin containing at least 32 units per mg. beginning on the day of operation (4th day).

† Since two rats died on the 20th day the figure for the change in weight between the 4th and 19th days is given.

‡ Each rat received 17 daily injections of the stated dose beginning on the day of operation (4th day).

*Relation between the intensity of lactation in intact rats and the degree of inhibition due to adrenalectomy*

The results of a number of separate experiments indicated that the degree of lactational inhibition due to adrenalectomy varied somewhat from one experiment to another. Similar variations, usually relatively slight, in the lactational performance of the

control groups were also apparent and suggested a possible relation between the initial level of lactation and the severity of inhibition due to adrenalectomy.

In Table 2 are given the litter-growth indices [see Cowie & Folley, 1946*b*] for control rats together with the corresponding values for adrenalectomized rats expressed as percentages of the control values obtained in five experiments, in each of which, as is our usual practice, rats were chosen at random for adrenalectomy or sham-operation on the 4th day of lactation from a batch of normally lactating females.

Table 2. *Relation between the intensity of lactation in the rat and the degree of inhibition due to adrenalectomy*

Control rats			Adrenalectomized rats				
No. of rats in group	Mean no. of pups per litter over days 6-11	Litter-growth index g./day*	No. of rats in group	Mean no. of pups per litter over days 6-11	Litter-growth index		Per cent of original pups alive on day 16
					g./day	% of control	
9	8.0	16.7	9	7.9	6.7	40.1	99.0
6	7.5	15.3	6	7.3	5.9	38.6	98.0
5	7.8	14.6	6	7.3	5.0	34.3	84.5
6	7.8	13.7	6	7.5	3.7	27.0	61.0
4	8.0	12.2	6	7.5†	Very small	Very small	0

\* The litter-growth index of a group of rats is defined as the mean daily gain in weight per litter over the 5-day period from the 6th to the 11th days [Cowie & Folley, 1946*b*].

† Calculated over days 6-8 since numerous deaths among the litters occurred from day 10 onwards.

It is apparent that there is a negative correlation between the lactational performance of the controls and the degree of lactational failure due to adrenalectomy. This conclusion is in general confirmed by the figures for the survival rates of the litters of the adrenalectomized groups at the 16th day, given in the last column of Table 2. Survival rates at the 16th day, and to a lesser extent at weaning (21st day), are useful as a secondary criterion of the degree of lactational failure in cases where the latter is of such a degree that the young begin to die before the 16th day.

The litter-growth indices of the various groups of control rats, given in Table 2, illustrate the variation in the intensity of lactation which occurs in our rats from time to time. In the experiment in which the lactational performance of the control rats was the lowest observed in the present series of experiments, the effects of adrenalectomy were so severe (see Table 2 and Fig. 4) as to amount to almost complete inhibition, all the young of the adrenalectomized rats being dead by the 14th day, i.e. 10 days after the operation. The shape of the litter-growth curve for this group (Fig. 4) was such that it was impossible to determine the litter-growth index and for comparative purposes it was assumed to be about zero. This is the only experiment so far in which we have observed such severe effects of adrenalectomy upon lactation—effects much more severe than those observed by Gaunt *et al.* [1942].

#### *Effects of deoxycorticosterone acetate on lactation in adrenalectomized rats*

An experiment was carried out in which in addition to the usual groups of sham-operated and adrenalectomized animals, groups of adrenalectomized rats received 0.1, 1.0 or 3.0 mg. of deoxycorticosterone acetate daily from the 4th to the 20th days

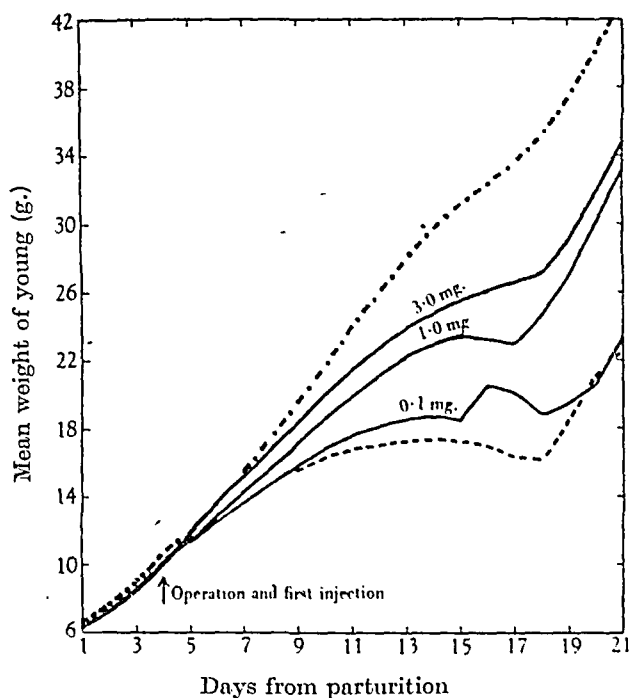


FIG. 2. Effect of various doses of deoxycorticosterone acetate on lactation in adrenalectomized rats. The continuous lines are mean growth curves of litters of groups of adrenalectomized rats receiving the indicated daily doses of deoxycorticosterone acetate. The upper and lower broken lines are mean growth curves of litters of groups of sham-operated and untreated adrenalectomized rats respectively.

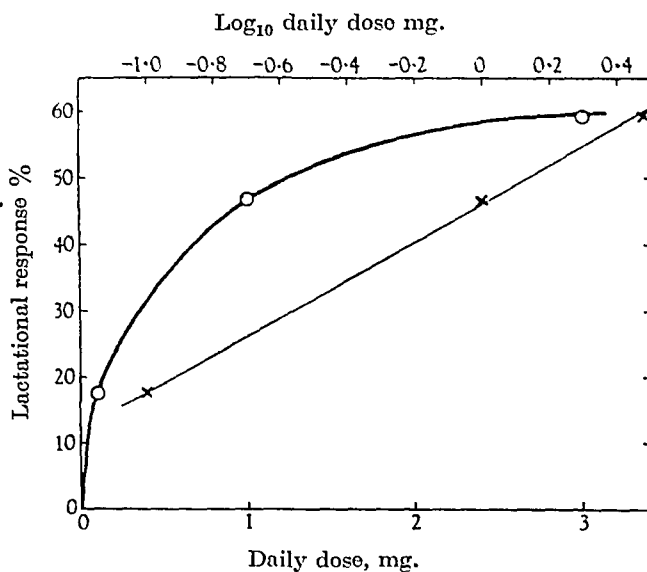


FIG. 3. The influence of daily injections of deoxycorticosterone acetate from the 4th (day of operation) to the 21st day of lactation on lactation in adrenalectomized rats. O—O, relation between the lactational response and the daily dose. x—x, relation between the lactational response and the logarithm of the daily dose. The response is given by  $\frac{100(c-b)}{a-b}$ , where  $a$ ,  $b$  and  $c$  are respectively the mean daily increase in weight per litter for control rats, untreated adrenalectomized rats, and treated adrenalectomized rats, in each case calculated over the 5-day period from the 6th to the 11th days of lactation.

inclusive. The results which are shown in Fig. 2 (see also Table 1) confirm and extend our previous finding that there is a graded relation between dose and response over this dose range.

Responses calculated as described by Cowie & Folley [1946*b*] are plotted against dose and also against  $\log_{10}$  dose in Fig. 3. In the first instance the points lie on a smooth curve, while in the second they give an excellent fit to a straight line, in this respect resembling many other dose-response relations. It must be remembered (*a*) that the response calculated in this way can only be regarded as an approximation since the litter-growth index gives only an approximate measure of the true milk yield, and (*b*) that no account has been taken of the possibility that, particularly in the case of the highest dosage, excess deoxycorticosterone acetate may find its way into the milk and directly affect the well-being and growth of the young. Nevertheless the regularity of the results obtained in this experiment is noteworthy and suggests that this response might be used as the basis of a method of assay of adrenal cortex extracts using deoxycorticosterone acetate as a standard.

The results in Fig. 3 suggest that the maximum response obtainable is about 60% of the theoretical. However, since the severity of the effects of adrenalectomy on lactation has been shown to vary from one experiment to another and in the same sense as the lactational efficiency of the sham-operated controls, it is of interest to see if the response to a given daily dose of deoxycorticosterone acetate shows similar

Table 3. *Effect of deoxycorticosterone acetate (doca) on lactation in adrenalectomized rats*

(Mean litter size in parentheses)

Daily dose of doca mg.	No. of rats in			Litter-growth index* of			
	Control group	Adrenal-ectomized group	Treated group	Control rats (a) g./day	Adrenal-ectomized rats (b) g./day	Adrenal-ectomized rats treated with	Response $\left(\frac{100(c-b)}{a-b}\right)$ %
						doca (c) g./day	
0.1†	9 (8.0)	9 (7.9)	5 (8.0)	16.7	6.7	9.4	27.0
0.1‡	6 (7.5)	6 (7.3)	6 (7.3)	15.3	5.9	7.5	17.0
1.0†	9 (8.0)	9 (7.9)	6 (7.9)	16.7	6.7	11.5	48.0
1.0‡	6 (7.5)	6 (7.3)	5 (7.2)	15.3	5.9	10.3	46.7
3.0†	6 (7.5)	6 (7.3)	6 (7.5)	15.3	5.9	11.5	59.6
3.0‡	6 (7.8)	6 (7.5)	6 (8.0)	13.7	3.7	11.5	78.0
3.0‡	4 (8.0)	6 (7.5)§	4 (7.0)	12.2	Very small	12.3	c. 100.0

\* The litter-growth index of a group of rats is defined as the mean daily gain in weight per litter over the 5-day period from the 6th to the 11th days [Cowie & Folley, 1946*b*].

† Each treated rat received 10 daily doses beginning on the 4th day of lactation, i.e. the day of operation.

‡ Each treated rat received 17 daily doses beginning on the 4th day of lactation, i.e. the day of operation.

§ Calculated over days 6-8 since numerous deaths among the litters occurred from day 10 onwards.

variations from time to time. The results of three experiments involving the administration of 3.0 mg. of deoxycorticosterone acetate daily are given in Table 3, from which it appears that the response to this dosage varied directly with the degree of inhibition and inversely as the efficiency of lactation in the controls. The supposed

maximum response of *c.* 60 % was well exceeded in the second of these three experiments and in the third, the exceptional experiment where inhibition was complete, the response was estimated as being approximately 100 %, an estimate confirmed by the growth curves shown in Fig. 4. The break in the growth curve of the control rats (Fig. 4) at the 18th day is of no practical significance; it was due to the inadvertent failure to feed three of the rats in this group during 2 days preceding weaning. In

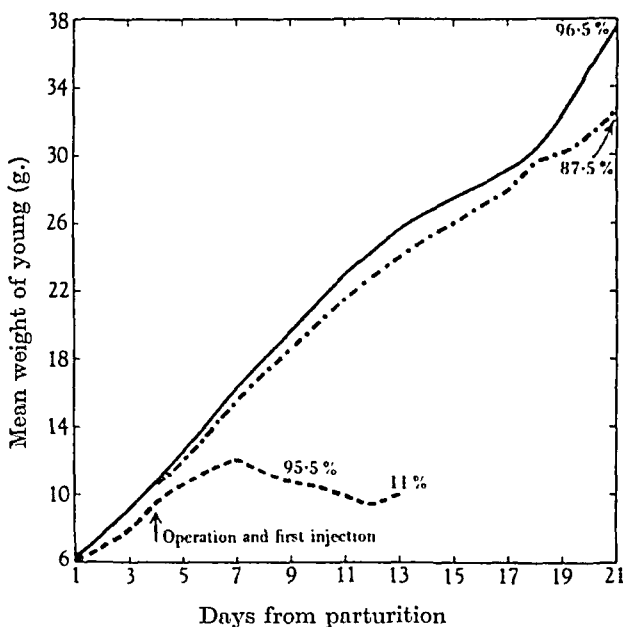


FIG. 4. Complete maintenance of lactation in a group of adrenalectomized rats receiving 3.0 mg. of deoxycorticosterone acetate daily from the 4th (day of operation) to the 21st days of lactation. The continuous line is the mean growth curve of the litters of a group of four adrenalectomized rats (28 pups at the 4th day) receiving deoxycorticosterone acetate. The upper and lower broken lines are mean growth curves of litters of groups of four sham-operated controls (32 pups at the 4th day) and six untreated adrenalectomized rats (45 pups at the 4th day) respectively. The figures near the curves give the percentages of pups surviving.

the two experiments in which 0.1 mg. of the steroid was given daily the response, on the other hand, varied inversely as the degree of inhibition due to adrenalectomy.

Clearly the results shown in Table 3 imply considerable variations from one experiment to another in the slope of the dose-response curve.

#### DISCUSSION

We have seen that while in our rats the degree of inhibition of lactation due to adrenalectomy on the 4th day may vary from time to time, the inhibition is in most cases only partial. On the average the adrenalectomized mothers seem capable of rearing almost 50 % of their young to a weaning weight about 50 % of the normal for this colony. In one exceptional experiment, however, the inhibition appeared to be complete or very nearly so, though it is improbable that secretion ceased entirely, since Levenstein [1936-7] found histological evidence of secretion in adrenalectomized rats which had lost their litters. In the experiments of Gaunt *et al.* [1942] also, the average body weight of the young of adrenalectomized rats was 50 % of that of control young at the 17th day, but since the survival rate at this time was

only about 45% as compared with our figure of 88% (see Fig. 1) the effects of adrenalectomy in their rats may be taken as somewhat more severe than are generally observed in ours [see also Folley & Cowie, 1944].

It is considered unlikely that the partial nature of the lactational inhibition is due to the regular presence of accessory adrenal cortex tissue in our rats for the following reasons. The amount of accessory tissue is not likely to vary from one batch of rats to another, particularly to such an extent that an exceptional batch of rats may have none as our results would make it necessary to assume. Further, survival experiments not yet published, which we have carried out on rats from the same colony, show that after adrenalectomy all save a small proportion regularly die within a predictable interval which varies slightly with the age of the rats used. The small proportion which survive apparently indefinitely, presumably possess accessory adrenal cortex tissue though it has not always been found on macroscopic inspection at autopsy. Finally, the fact that administration of adrenotrophin did not improve lactation in adrenalectomized rats, though not conclusive by itself, lends support to the belief that no accessory tissue is regularly present.

For the same reasons it is unlikely that the difference between our results and those of Gaunt *et al.* [1942], who observed more complete lactational inhibition than we did, could be due to differences in the amount of accessory adrenal cortex tissue possessed by our respective strains of rat. It remains possible that the differences may have been due to differences in the sodium and perhaps potassium, contents of our respective stock colony diets since lactational deficiencies in adrenalectomized rats may be slightly alleviated by administration of sodium chloride [Gaunt & Tobin, 1936; Levenstein, 1936-7; Folley & Cowie, 1944]. The variations in lactational efficiency which we have observed among groups of adrenalectomized rats in our own experiments cannot, however, be accounted for on this basis since the composition of the stock diet in this colony is kept constant. It seems more likely that these variations are connected in some way with the variations in the efficiency of lactation we have observed among our control groups. Analysis of all the data in our possession has failed to support our previous suggestion [Folley & Cowie, 1944], that such changes in lactational efficiency in the control groups may be connected with the seasonal variations described previously [Folley, Ikin, Kon & Scott Watson, 1938] in the proportion of parturient rats in this colony which totally fail to rear their young. The far slighter variations in lactational efficiency we are discussing here may possibly have as their immediate cause alterations in a delicately poised balance between factors which stimulate lactation (possibly anterior-pituitary hormones) and factors which tend to inhibit it. The latter role might well be filled by oestrogen which, as is well known, can inhibit lactation under certain conditions [see Folley, 1941] and it may be significant in the present connexion that the inhibitory action of a given dose of oestrogen is far more pronounced in adrenalectomized than in intact rats [Gaunt *et al.* 1942; Folley & Cowie, 1944]. Such a mechanism might thus provide an explanation of the observed inverse correlation between the lactational performance of the controls and the degree of inhibition due to adrenalectomy.

Our previous finding that deoxycorticosterone acetate will restore lactation to a considerable degree after adrenalectomy has been confirmed and we have now obtained further evidence that in any one experiment the response is regularly



related to the dose. In different experiments the response to a daily dose of 3.0 mg., calculated by a method which takes account of the lactational performance of intact and adrenalectomized controls, has amounted to a restoration of the lactational deficit due to loss of the adrenals varying from 60 to 100 %. It may be worth noting here that the rats used in the experiment in which deoxycorticosterone acetate gave a 100 % response differed from those used in all other experiments reported here and previously [Folley & Cowie, 1944] in that they were appreciably older (approx. 10 months old) and were undergoing their second lactation when used for experiment.

Claims to have demonstrated the existence of a specific lactation hormone (cortilactin) in the adrenal cortex [Brownell, Lockwood & Hartman, 1933; Spoor, Hartman & Brownell, 1941] are difficult to reconcile (*a*) with the fact that in one experiment deoxycorticosterone acetate gave complete restoration of lactation, and (*b*) with the complete restoration with 17-hydroxy-11-dehydrocorticosterone reported by Gaunt *et al.* [1942].

We do not propose to speculate at present on the possible reasons for variations in the lactational response of adrenalectomized rats to deoxycorticosterone acetate observed in these experiments, nor to comment further on the fact that while at the 3.0 mg. level the response varied directly with the degree of inhibition, the opposite was the case in two experiments in which 0.1 mg. was administered. It may be pointed out, however, that if the absolute level of lactation attained by the rats receiving 3.0 mg. had been taken as a measure of the response, the latter would have shown little variation in all three experiments. Nevertheless it would seem that our method of estimating the response [Cowie & Folley, 1946*b*], taking into account the lactational performance of intact and adrenalectomized controls run at the same time as the treated group, has the sounder theoretical basis.

#### SUMMARY

1. Adrenalectomy on the 4th day of lactation causes a marked, but usually only partial, inhibition of lactation in our rats. Variations in the degree of inhibition from one experiment to another have been observed and in one experiment inhibition was complete or nearly so.

2. There was a marked negative correlation between the degree of inhibition due to adrenalectomy and the lactational performance of sham-operated controls, which itself varied somewhat from one experiment to another.

3. The fact that in most cases adrenalectomy does not entirely abolish lactation is not due to the presence in our rats of accessory adrenal cortex tissue.

4. Lactation was maintained, in most cases partially, in adrenalectomized rats by daily administration of deoxycorticosterone acetate. Over the dose range studied there was a graded relation between dose and response. The relation between log dose and response over this range appeared to be linear.

5. The lactational response to a given dose of deoxycorticosterone acetate has been found to vary widely from one experiment to another; the response to 3.0 mg. daily varied directly with the degree of lactational inhibition due to adrenalectomy. In one experiment in which inhibition in untreated adrenalectomized rats was, to all intents and purposes, complete, lactation was fully restored to the control level by 3.0 mg. of deoxycorticosterone acetate daily.

We are greatly indebted to Dr S. K. Kon for generously placing at our disposal the facilities of the rat colony maintained by him at this Institute for nutritional investigations, and to Dr M. Reiss for a supply of standardized adrenotrophin. Our best thanks are also due to Dr A. N. Macbeth of Organon Laboratories, Ltd., for generous supplies of deoxycorticosterone acetate and to Mr W. S. Ferguson of Messrs I.C.I. Ltd., Jealott's Hill Research Station, for determining the  $\text{Na}_2\text{O}$  and  $\text{K}_2\text{O}$  contents of our stock colony diet. We are happy to acknowledge the help of the Agricultural Research Council who provided one of us (A.T.C.) with a special research grant.

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# ADRENALECTOMY AND REPLACEMENT THERAPY IN LACTATING RATS

## 3. EFFECTS OF DEOXYCORTICOSTERONE ACETATE AND 11-OXYGENATED CORTICAL STEROIDS ON LACTATION IN ADRENALECTOMIZED RATS MAINTAINED ON STOCK OR HIGH-PROTEIN DIETS

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In the second paper of this series [Cowie & Folley, 1946*a*] we have shown that in our rats the inhibition of lactation due to adrenalectomy on the 4th day is in general not complete, though in one experiment the effects of adrenalectomy were so severe as to amount to complete inhibition. The lactational deficiency resulting from adrenalectomy could be restored to an extent varying between 60% and 100% by administration of 3.0 mg. of deoxycorticosterone acetate daily. Earlier, Gaunt, Eversole & Kendall [1942] had reported that 17-hydroxy-11-dehydrocorticosterone (Kendall's compound E) and 11-dehydrocorticosterone (Kendall's compound A) were more effective than deoxycorticosterone acetate in maintaining lactation in adrenalectomized rats. Complete lactational replacement was afforded by compound E and by an extract of adrenal cortex while the response to deoxycorticosterone acetate, besides being less marked, appeared to be unrelated to the dose. Gaunt *et al.* thus concluded that for maintenance of lactation in adrenalectomized rats the restoration of a normal electrolyte metabolism may be helpful but not self-sufficient, the critical factor for maximal secretion being a sufficiency of those substances, the 11-oxygenated cortical steroids, the principal effects of which are concerned with protein and carbohydrate metabolism [see Kendall, 1941; Swingle & Remington, 1944].

As stated above, however, we have found deoxycorticosterone acetate consistently to produce considerable improvement in lactation in our adrenalectomized rats and we have found that the response bears a regular relation to the dose [Cowie & Folley, 1946*a*]. Moreover, in an earlier study [Folley & Cowie, 1944] of the comparative effects of equal doses of deoxycorticosterone acetate and of compounds A and E on lactation in adrenalectomized rats, we had found the former substance to be more effective than either of the other two. In fact the effect of compound A appeared to be, if anything, deleterious.

In seeking reasons for the evident discrepancy between our results and those of Gaunt *et al.* [1942] the possibility that dietary factors are involved at once comes to mind. Thus it is known that the intake of sodium and potassium may affect the survival of adrenalectomized rats (references in Swingle & Remington [1944]) and a diet containing unfavourable proportions of these elements might conceivably prevent 11-oxygenated cortical steroids from correcting lactational deficiencies due to adrenalectomy, assuming, of course, that the views of Gaunt *et al.* on the main cause of these

deficiencies are correct. Another, and even more attractive, possibility is opened up by the work of Olsen, Jacobs, Richert, Thayer, Kopp & Wade [1944] who found that 11-oxygenated cortical steroids caused more glycogen deposition in the livers of adrenalectomized rats maintained on high-protein diets (approximately 50 % protein) than occurred in rats receiving diets containing more usual (*c.* 20 %) proportions of protein. It thus seemed possible that if the crucial physiological disturbance responsible for the lactational inhibition following adrenalectomy is a disturbance of protein and carbohydrate metabolism due to loss of those factors concerned with the promotion of gluconeogenesis, these factors, the 11-oxygenated cortical steroids, might prove superior to deoxycorticosterone acetate in maintaining lactation in adrenalectomized animals receiving a high-protein diet thus ensuring a plentiful supply of circulating amino acids for deamination.

The experiments described in this paper were carried out with two objects in view. First, to confirm our previous findings as to the relative effectiveness of deoxycorticosterone acetate and compounds A and E in maintaining lactation after adrenalectomy when giving smaller doses of compounds A and E than before (since the results then suggested that perhaps too much compound A was being given) and such larger doses of deoxycorticosterone acetate as were subsequently found to be most effective [Cowie & Folley, 1946*a*]. Secondly, to examine the effect on the relative effectiveness of the two types of cortical hormone, of raising to approximately 50 % the protein content of the diet of the test animals.

## EXPERIMENTAL

### *Methods*

The rats (uniparous females approximately 5 months old), and the general experimental procedure were as previously described [Folley & Cowie, 1944; Cowie & Folley, 1946*a*]. The compositions of the stock diet (diet 5) and the high-protein diet (diet 253) are given in Table 1. Diet 253, containing approximately 50 % protein, was derived from

Table 1. *Composition of stock diet (diet 5) and high-protein diet (diet 253)*

	Diet 5	Diet 253
	%	%
Whole wheat	69.0	19.5
Linseed cake	14.0	14.0
Crude casein	6.0	50.0
Dried brewers' yeast	5.0	10.0
Margarine	5.0	5.0
CaCO <sub>3</sub>	0.5	1.0
NaCl	0.5	0.5
Na <sub>2</sub> O on moisture-free basis (by analysis)	{ 0.40 0.44*	0.49*
K <sub>2</sub> O on moisture-free basis (by analysis)	{ 0.95 1.06*	0.96*

\* Values obtained after addition to basal diet of whole dried milk in the ratio of 375/1000 by weight.

diet 5 by increasing the content of casein and dried yeast at the expense of the whole wheat. Both diets were fed *ad lib.*, rats on diet 5 receiving in addition, as in previous experiments, cows' milk *ad lib.* and approximately 5 g. of raw liver twice weekly and raw carrot daily except Sundays. In order to ensure that rats on diet 253 would

consume as nearly as possible the same proportions of basal diet and milk as rats on diet 5, the former were given no liquid milk; instead whole dried milk was mixed with the basal diet in the ratio 375 : 1000 which has been found to correspond to the average proportions of milk and diet 5 consumed by stock colony rats. These rats also received the above-mentioned supplements of raw liver and carrot. Rats randomly selected to receive diet 253 were placed on this diet at parturition. It will be seen from the figures (Table 1) for the  $\text{Na}_2\text{O}$  and  $\text{K}_2\text{O}$  contents of diet 5, with and without the addition of dried milk, that the milk supplement however fed did not significantly alter the sodium and potassium intake.

As before, adrenalectomy was performed on the 4th day of lactation, controls being subjected to a sham operation. The three steroids were administered once daily by subcutaneous injection, dissolved in sesame oil so that the daily dose was contained in 0.5 ml., 17 injections, beginning on the day of operation, being given in each case. Owing to scarcity of material, compound E could be administered to one group only, maintained on diet 253. Controls received appropriate injections of sesame oil. Compounds A and E are rather sparingly soluble in oil so stock solutions of all three steroids were made up in acetone. Each day just sufficient solution was added to sesame oil and the acetone removed at 70–80° C. in a current of air. The solutions of compounds A and E thus prepared were supersaturated but did not precipitate out before use. The sesame oil used for the control injections was similarly treated. A commercial extract of adrenal cortex (Eucortone) was also used; this was injected twice daily and these rats also received control injections of sesame oil.

### Results

Table 2 gives the mean weights and survival rates of the young of the various groups at the 4th, 16th and 21st days. The litter-growth indices and the lactational responses calculated by the methods of Cowie & Folley [1946*b*] are given in Table 3. In cases (three in all) where a litter suffered reduction during the 5-day period over which the litter-growth indices were calculated, the data for this litter were omitted from the calculation since the latter involves the total litter weight, not the average weight of each pup. In two cases where only one pup died this made little difference to the results but in the case of the group on diet 253 receiving compound A, one member of which lost two pups, this correction made an important difference.

In the first place it is evident that the high-protein diet exerted an unfavourable effect on lactation in intact rats, but in adrenalectomized rats, on the other hand, there was very little difference in lactational performance between groups receiving either diet.

In comparing the lactational performance of groups of rats between which the difference is slight or nil, it seems best not to rely only on the litter-growth indices but to take into consideration also the survival rates and mean growth curves of the litters, remembering that the second of these additional criteria is dependent to some extent on the first. For the death of a good proportion of the young of a group of feebly lactating rats will cause a sharp rise in the depressed growth curve since the survivors are thereafter better nourished. In the present instance the litter-growth indices (Table 3) indicate that of the untreated adrenalectomized rats, those on diet 253 lactated the better but only slightly so; survival rates and weaning weights

Table 2. *Effect of adrenal cortex steroids on lactation in adrenalectomized rats maintained on the stock diet (diet 5) and a high-protein diet (diet 253)*

Treatment	Daily dose mg.	No. of mothers	Total no. of young on 4th day		Mean wt. (g.) of young on day			Per cent of young alive on day			No. of mothers dead by 21st day	No. of litters dead by 21st day	Per cent change in wt. of mothers between 4th and 21st days
			♂	♀	4	16	21	4	16	21			
(a) Rats on diet 5													
Sham operation:													
No treatment	—	6	25	22	10.5	30.5	42.0	98	98	98	0	0	+ 8.8
Adrenalectomy:													
No treatment	—	6	22	23	10.0	16.0	21.0	98	61	35	0	3	— 9.1
Deoxycorticosterone acetate	3.0	6	24	24	10.1	25.5	34.0	100	100	98	0	0	+16.3
11-Dehydrocorticosterone	0.5	6	24	23	10.5	18.2	24.0	100	96	64	0	2	— 8.8
Adrenal cortex extract	2 × 0.375 (ml.)	6	23	24	10.7	20.0	25.9	100	100	96	0	0	— 0.4
(b) Rats on diet 253													
Sham operation:													
No treatment	—	6	23	24	9.0	23.6	31.0	100	100	100	0	0	+ 6.7
Adrenalectomy:													
No treatment	—	6	24	23	9.7	14.4	17.6	100	94	26	1	4	—10.5
Deoxycorticosterone acetate	3.0	6	23	23	9.5	22.1	29.2	98	98	98	0	0	+22.5
11-Dehydrocorticosterone	0.5	6	24	24	9.9	16.0	17.5	100	90	42	1	2	—11.3
17-Hydroxy-11-dehydrocorticosterone	0.47	5	21	19	9.7	16.8	17.5	100	80	70	1	1	—10.3
Adrenal cortex extract	2 × 0.375 (ml.)	6	24	24	9.8	16.6	17.6	100	100	90	0	0	— 0.9

Table 3. *Lactational responses to adrenal cortex steroids of adrenalectomized rats maintained on the stock diet (diet 5) or on a high protein diet (diet 253)*

Treatment	Dose mg.	No. of mothers	Mean no. of young per litter over days 6-11	Litter-growth index* g./day	Response† %
(a) Rats on diet 5					
Sham operation:					
No treatment	—	6	7.8	13.7	—
Adrenalectomy:					
No treatment	—	6	7.5	3.7	—
Deoxycorticosterone acetate	3.0	6	8.0	11.5	78.0
11-Dehydrocorticosterone	0.5	6	7.8	7.0	33.0
Adrenal cortex extract	2 × 0.375 (ml.)	6	7.8	8.0	43.0
(b) Rats on diet 253					
Sham operation:					
No treatment	—	6	7.8	10.3	—
Adrenalectomy:					
No treatment	—	5	7.6	4.4	—
Deoxycorticosterone acetate	3.0	6	7.7	8.5	69.5
11-Dehydrocorticosterone	0.5	5	8.0	5.0	10.2
17-Hydroxy-11-dehydrocorticosterone	0.47	4	8.0	5.0	10.2
Adrenal cortex extract	2 × 0.375 (ml.)	6	8.0	4.8	6.8

\* The litter-growth index of a group of rats is defined as the mean daily gain in weight per litter over the 5-day period from the 6th to the 11th days [Cowie & Folley, 1946b].

† The response is given by  $\frac{100(c-b)}{a-b}$ , where  $a$ ,  $b$  and  $c$  are respectively the litter-growth indices of the groups of control, untreated adrenalectomized and treated adrenalectomized rats [Cowie & Folley, 1946b].

(Table 2) on the other hand suggest the reverse. The lactation curves (Fig. 1) lie very close together, however, and it seems safe to conclude that adrenalectomy abolishes the difference between the two diets in respect of their capacities for supporting lactation.

Our previous finding [Folley & Cowie, 1944] regarding the superiority of deoxycorticosterone acetate over compounds A and E in maintaining lactation after adrenalectomy has now been confirmed under different conditions of dosage, the response in rats on diet 5 to 3.0 mg. of deoxycorticosterone acetate daily being 78 % as against a 33 % response in rats receiving 0.5 mg. of compound A daily (Table 3). It should

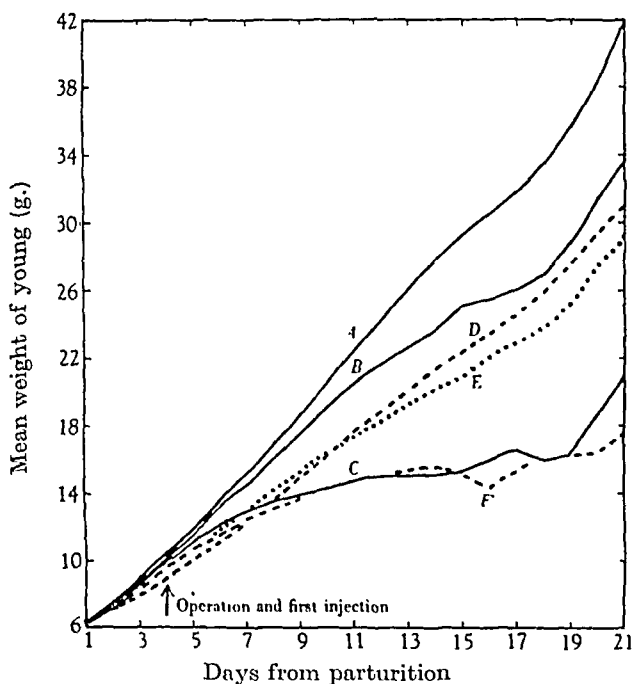


FIG. 1. Effect of adrenalectomy and replacement therapy with deoxycorticosterone acetate on lactation in rats maintained on a stock diet (diet 5) or a high-protein diet (diet 253). The curves shown represent the mean growth curves of the litters of groups of rats. *A*, sham-operated rats on diet 5; *B*, adrenalectomized rats on diet 5 receiving 3.0 mg. of deoxycorticosterone acetate daily; *C*, untreated adrenalectomized rats on diet 5; *D*, sham-operated rats on diet 253; *E*, adrenalectomized rats on diet 253 receiving 3.0 mg. of deoxycorticosterone acetate daily; *F*, untreated adrenalectomized rats on diet 253.

be noted that the daily dose of deoxycorticosterone acetate was six times that of compound A but that the response to the latter was now positive (see also Fig. 2), whereas previously we had observed a deleterious effect with twice the present dose. The adrenal cortex extract in the dosage used was also much less effective than deoxycorticosterone acetate (Tables 2 and 3) as we had previously found with a different extract. The same dose of deoxycorticosterone acetate gave almost as great a response in rats on the high-protein diet (Fig. 1 and Table 3) but in these rats the responses to compound A and the adrenal cortex extract were much reduced.

The growth curves (Figs. 2, 3)\* indicate that on the high-protein diet there was little to choose between the lactational performances of adrenalectomized rats receiving compound A, adrenal cortex extract, or no treatment at all, but the calculated responses (Table 3) were positive in the treated rats and amounted to 10.2% and 6.8 %

\* Note that in Figs. 2 and 3 the vertical scale is twice that in Fig. 1.

respectively. However, even if we attach more evidential weight to the responses than to the close coincidence of the growth curves and conclude that the two treatments in question did in fact exert a slight beneficial effect in high-protein fed adrenalectomized rats, the contrast between the responses to these treatments of rats on diets 5 and 253 respectively is obvious. The response of adrenalectomized rats on the high-protein diet to compound E was, as far as can be seen (Tables 2 and 3; Figs. 2, 3), of the same order as the response to compound A and since we had

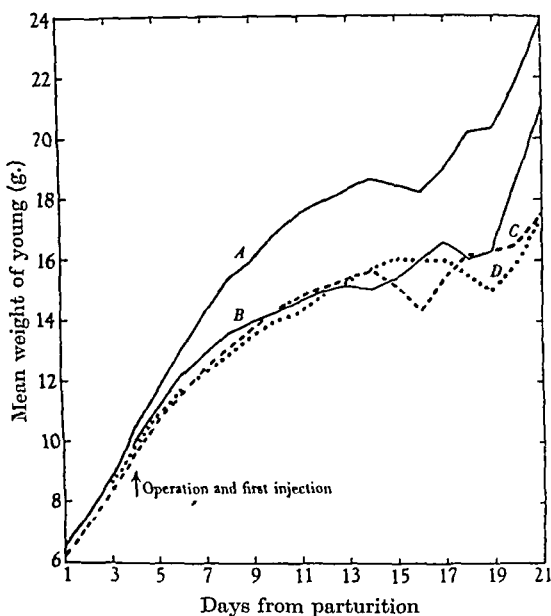


FIG. 2.

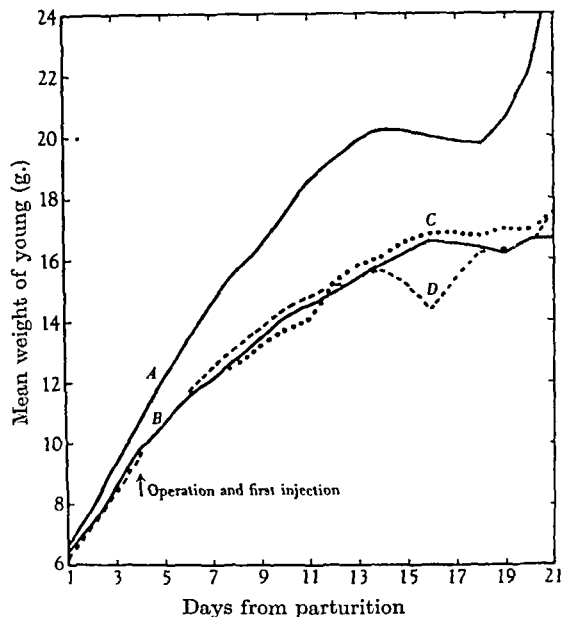


FIG. 3.

FIG. 2. Effect of 11-dehydrocorticosterone (compound A) on lactation in adrenalectomized rats maintained on a stock diet (diet 5) or a high-protein diet (diet 253). The curves shown represent the mean growth curves of the litters of groups of rats. A, adrenalectomized rats on diet 5 receiving 0.5 mg. of compound A daily; B, untreated adrenalectomized rats on diet 5; C, untreated adrenalectomized rats on diet 253; D, adrenalectomized rats on diet 253 receiving 0.5 mg. of compound A daily.

FIG. 3. Effect of 17-hydroxy-11-dehydrocorticosterone (compound E) and adrenal cortex extract on lactation in adrenalectomized rats maintained on a stock diet (diet 5) or a high-protein diet (diet 253). The curves shown represent the mean growth curves of the litters of groups of rats. A, adrenalectomized rats on diet 5 receiving 0.375 ml. of adrenal cortex extract twice daily; B, adrenalectomized rats on diet 253 receiving 0.375 ml. of adrenal cortex extract twice daily; C, adrenalectomized rats on diet 253 receiving 0.47 mg. of compound E daily; D, untreated adrenalectomized rats on diet 253.

previously found [Folley & Cowie, 1944] that weight for weight the former was more effective than the latter in maintaining lactation after adrenalectomy it is reasonable to infer that had supplies permitted a comparison of the effectiveness of compound E in adrenalectomized rats receiving both diets, a reduced response in rats on the high-protein diet would have been found in this case also.

The effect of deoxycorticosterone acetate on the body weight of the mothers is noteworthy (Table 2). Control groups on both diets made slight gains in weight between the operation and the day of weaning. In the groups receiving compounds A and E, irrespective of diet, the weight losses were considerable and of the same order as those of untreated adrenalectomized rats, while the changes in weight of the rats



receiving adrenal cortex extract were negligible. In contrast with these results it will be seen that both groups of rats receiving deoxycorticosterone acetate gained far more weight even than the controls. These results are reminiscent of those found for the effects of various adrenal cortex steroids on the growth of immature adrenalectomized rats [Wells & Kendall, 1940].

#### DISCUSSION

The present results indicate that our rats did not lactate so well on the high-protein diet containing approximately 50 % protein as on the stock diet containing approximately 20 %. This finding confirms previous work [Slonaker, 1931; McCoy, 1940] which indicated that high-protein diets are incompatible with optimal lactation.

As in our previous experiments [Folley & Cowie, 1944], deoxycorticosterone acetate appears to maintain lactation in adrenalectomized rats receiving the stock diet better than 11-oxygenated cortical steroids or such extracts of adrenal cortex as have been available to us. It is noteworthy, and perhaps adds weight to the above finding, that the comparison was now made under different conditions from before. The daily dose of deoxycorticosterone acetate used now was six times that of compound A, the dose of which was reduced in the present experiments to half that used previously by ourselves and found most efficacious by Gaunt *et al.* [1942]. Under the present conditions compound A gave a positive response in adrenalectomized rats on the stock diet whereas previously the effect of twice the present dose was distinctly deleterious. It may be recalled that our previous results with compound A had led us to suggest that doses lower than 1.0 mg. daily might give better results and so it has proved. It therefore seems possible that there may be a restricted optimum dose range for the maintenance of lactation in adrenalectomized rats by 11-oxygenated steroids. There is little doubt that, as far as present knowledge goes, in our rats and under our conditions deoxycorticosterone acetate is superior to 11-oxygenated steroids in maintaining lactation after adrenalectomy.

In adrenalectomized rats on the high-protein diet, while the absolute lactational performance of rats receiving 3.0 mg. of deoxycorticosterone acetate daily was inferior to that of corresponding recipients of the stock diet, the lactational response was of the same order; in other words the protein content of the diet had little effect on the effectiveness of this steroid. The results obtained with compounds A and E and the adrenal cortex extract stand in sharp contrast. Consideration of the evidence provided by the lactational responses (Table 3), the survival rates (Table 2) and the growth curves (Figs. 2, 3 and Table 2) suggests that if there was any response at all to compounds A and E and the cortical extract in rats on the high protein diet it must have been very small. There can be no doubt, therefore, that the high-protein diet, far from increasing, actually decreased the effectiveness of compound A and the adrenal cortex extract and by inference that of compound E also.

It is difficult to escape the conclusion, therefore, that in our rats and under our conditions the primary defect in lactation resulting from loss of the adrenals is due to interference with some physiological mechanism which is reinstated to a considerable extent by hormones of the deoxycorticosterone class, the effects of which according to current theories [see Kendall, 1941; Swingle & Remington, 1944] seem to be more particularly concerned with electrolyte metabolism. If the crucial necessity for normal lactation had been an adequate supply of cortical hormones more specifically con-

cerned with protein and carbohydrate metabolism it would have been expected that 11-oxygenated steroids would have been more rather than less effective in the presence of a high-protein intake with the resulting increased supply of amino acids for deamination and conversion to sugar.

## SUMMARY

1. Rats receiving a high-protein diet (approximately 50 % protein) lactated less efficiently than rats on a stock diet (containing approximately 20 % protein). Adrenalectomized rats, however, lactated equally well on either diet, but of course at a much reduced level.

2. Deoxycorticosterone acetate was more effective than 11-dehydrocorticosterone or an adrenal cortex extract in maintaining lactation in adrenalectomized rats on the stock diet, thus confirming our previous findings under different conditions of dosage.

3. 11-Dehydrocorticosterone (0.5 mg. daily) gave a positive lactational response in adrenalectomized rats on the stock diet whereas we had previously observed a deleterious effect with 1.0 mg. daily.

4. The lactational response of adrenalectomized rats to deoxycorticosterone acetate was almost as great on the high-protein diet as on the stock diet, but the response to 11-oxygenated cortical steroids and adrenal cortex extract was very much reduced by the high-protein diet.

5. The evidence suggests that in our rats the primary cause of the lactational inhibition which follows adrenalectomy cannot be due to the loss of those adrenal cortex hormones, the 11-oxygenated steroids, which are closely concerned in protein and carbohydrate metabolism.

In connexion with this work we have had the advantage of discussion by correspondence with Dr E. C. Kendall and Dr Robert Gaunt. Dr Kendall, in addition to drawing our attention, before the paper of Olsen *et al.* [1944] arrived in this country, to possibilities in connexion with the use of high-protein diets, furnished generous supplies of compounds A and E and to him we express our sincere thanks. We are also indebted to Dr S. K. Kon for providing facilities for working with rats and for advice in connexion with the design of the high-protein diet. Our best thanks are also due to Dr A. N. Macbeth of Organon Laboratories, Ltd., for supplies of deoxycorticosterone acetate, to Mr C. J. Eastland of Messrs Allen and Hanbury Ltd. for the adrenal cortex extract, and to Mr W. S. Ferguson of Messrs I.C.I. Ltd., Jealott's Hill Research Station, for determinations of the sodium and potassium contents of our rat diets. We are happy to acknowledge the help of the Agricultural Research Council who provided one of us (A.T.C.) with a special research grant.

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# LATE EFFECTS OF CASTRATION AND ADMINISTRATION OF SEX HORMONES ON THE MALE *TRICHOSURUS VULPECULA*

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In a previous communication [Bolliger, 1944*a*] it had been shown in one experiment that by means of castration and subsequent administration of oestrogens the scrotum of *Trichosurus vulpecula* (Australian grey opossum, commonly called possum) is completely transformed into a pouch. This communication was confined to external observations and the experiment described in it was subsequently continued for another 18 months. Ultimately this animal and three others with a somewhat similar history were killed and in addition the internal changes subsequent to castration and oestrogen administration were examined. At the same time external and internal changes were examined in a male which had only been castrated.

## THE EFFECT OF CASTRATION ON THE ADOLESCENT MALE

*Animal and treatment.* A male of about 7 months of age weighing 720 g. was castrated (25 Sept. 1944), the testes without the epididymes being removed by two lateral incisions in the scrotum. The diameter of the removed testes was about 0.7 cm. There were no ill effects from the operation and the animal increased in weight more or less normally. On 26 Jan. 1945 the animal weighed 1.86 kg. The animal was killed on 20 Aug. 1945 when it weighed 2.18 kg. and appeared to be well developed although somewhat obese.

## Observations

*External.* The animal was killed about 11 months after castration when it was about 18 months old. It did not then possess the dark brown sternal hairs [Bolliger & Hardy, 1944] nor the reddish brown hairs over the scapular area which are usually found in normal males of this age. The dorsal pelage was of a grey colour similar to that seen in females.

The empty scrotum of the animal was about 2.8 cm. long and the maximum width at its base was 3.0 cm., thus forming an almost equilateral triangle. There was only slight evidence of infolding of the peri-scrotal skin and a definite pouch had not been formed (Pl. 1, fig. 1).

*Internal.* The subcutaneous fat and the fat about the muscles were definitely increased. The viscera appeared to be normal, apart from a marked increase in abdominal fat and the almost complete disappearance of the prostate. The prostatic remnant with the enclosed urethra was 0.4 cm. wide near the bladder neck and tapered down to 0.2 cm.; its total length was about 2.5 cm. and it weighed 0.35 g. None of the pigmentation characteristic of the normal prostate could be seen (Pl. 2, fig. 3). Normally the width of the prostate with enclosed urethra near the bladder neck is 2–3 cm., the normal length 5–6 cm., and the normal weight about 20 g. [Carrodus & Bolliger, 1939].

On microscopic examination the prostatic remnant near the cephalic end was found to consist mainly of a thick outer covering (about 0.15 cm. wide) composed of fibrous tissue interwoven with strands of involuntary muscle. The glandular portion of the original prostate had been almost completely replaced by fibrous tissue containing very small vestiges of atrophied tubules (Pl. 4, fig. 6). The centre of the prostate had a thinner outer covering. The parenchyma was also almost completely replaced by fibrous tissue, though a few ducts near the urethra were still in existence. In its whole length the urethral lining was raised into a number of longitudinal folds, the circular appearance seen on cross-section in the untreated animal having been lost owing to shrinkage of the urethral lumen. Thus, in cross-sectional area the entire lumen of the urethra was roughly only a tenth of that of the normal prostatic urethra. It was covered with stratified squamous epithelium instead of the columnar epithelium of the normal organ.

#### EFFECT OF CASTRATION AND OESTROGEN ON THE ADOLESCENT AND FULL-GROWN MALE

##### *Adolescent male*

##### *Animal and treatment*

Injections of stilboestrol were started when the animal was still living in the mother's pouch and was about 3 months old. The injections were continued until a total of 1.8 mg. had been given during 4 months. The animal was then castrated. Three injections of oestradiol dipropionate were given; one a month after castration and two 6 months later.

*Details.* 0.1 mg. of stilboestrol on 1, 15 and 23 March 1943.

0.2 mg. of stilboestrol on 12 April when the animal (450 g.) left the pouch.

0.3 mg. of stilboestrol on 22 April.

0.5 mg. of stilboestrol on 19 and 28 June.

Castration on 15 July.

0.5 mg. of oestradiol dipropionate on 19 Aug.

0.4 mg. of oestradiol dipropionate on 7 Feb. 1944.

0.7 mg. of oestradiol dipropionate on 18 Feb. 1944.

##### *Observations*

*External.* In February 1944, 6½ months after castration, the scrotum was considerably shrivelled up and two semilunar folds had formed on each side of the scrotal remnant, strongly suggesting the formation of the lips of a rudimentary pouch. After the injection of two further amounts of oestradiol dipropionate the scrotal remnant involuted further, and 1 month after the last injection a definite marsupial pouch of the diprotodont pattern had formed. At the same time it was noted that the animal was wet on its ventral surface, a condition met with in a number of normal and experimental animals, and probably due to excessive secretion by the skin glands.

The complete involution of the scrotum was noticed about 2 months after the last injection, and the capacity of the pouch [Bolliger, 1942a] was found to exceed 3 ml. The animal still remained wet on its ventral aspect. Furthermore, on account of the diminution in the length of the penis, the animal found difficulty in voiding urine in a stream. Contamination of the hind end of the animal by urine led to swelling and inflammation of the cloacal hillock.

During the subsequent 15 months the pouch remained intact, forming a caudal recess of 1–2 cm. in depth and a maximum width of 5–6 cm. The animal could approximate the lips of this artificial pouch. When relaxed the greatest distance between the pouch lips was 0.5–1.0 cm. The moisture on the ventral surface, though showing periods of remission, undoubtedly interfered with the general health of the animal.

On 28 August 1944 the animal weighed 1.82 kg. and was in fair condition. On 23 July 1945 it weighed only 1.52 kg. and was definitely emaciated. It was then killed. The sternal area [Bolliger & Hardy, 1944] consisted of light yellow-brown and grey hairs and was about 1 cm. wide and 3 cm. long, that is, about one-third of the normal area.

A definite pouch was present. Its inferior recess was 1.5 cm. deep, and the maximum depth of the lateral recesses was 2.5 cm. on each side. The pouch, though persistent in the dead animal, could be readily everted. In contrast to the experiment previously reported [Bolliger, 1944*a*] the pouch was symmetrical and the bilateral structure of the original scrotum was evidenced by slight symmetrical elevations on the floor of the pouch. The penis of the animal was still well formed although about half the size seen in the normal fully grown male.

*Internal examination.* The internal organs appeared to be normal except that the prostate was much reduced in size and showed no evidence of pigmentation (Pl. 2, fig. 4). Its length was 2.9 cm., its maximum width 0.9 cm., and it weighed 1.2 g. The normal weight would have been about 20 g.

On microscopical examination of a cross-section of the prostate, the urethral lumen was found to be branched. The lining epithelium had undergone metaplasia from a columnar, to a stratified squamous, type. The prostatic tubular vestiges were small, with the exception of some of moderate size adjacent to the urethral lumen. The tubular vestiges were lined by a stratified squamous epithelium, which had replaced the original cuboidal epithelium; in some the stratified squamous epithelium filled the lumen. Comparatively few of the tubules contained debris, and leucocytes were absent. The outer layer of the prostate was composed mainly of fibrous tissue. Thin strands of muscle tissue, which were observed throughout the substance of the prostate, were particularly numerous in this outer layer (Pl. 4, fig. 7).

#### *Animal and treatment*

#### *The full-grown male*

A young male was injected with 5.75 mg. of stilboestrol during 2 months following orchidectomy and was killed a year later. The animal was sexually mature when operated upon as shown by the presence of spermatazoa in the urine.

*Details.* Orchidectomy (epididymis left *in situ*) on 4 Feb. 1943.

0.5 mg. of stilboestrol on 15 Feb.

0.75 mg. of stilboestrol on 24 Feb.

1.0 mg. of stilboestrol on 4, 15 and 23 March.

1.5 mg. of stilboestrol on 22 April.

#### *Observations*

*External.* The recovery from orchidectomy was uneventful. The animal was off its food for about a week after the injection of this comparatively large amount of

stilboestrol. About a month after stopping the injections the scrotum was collapsed, forming a heap of wrinkled tissue, but no definite pouch had formed.

When killed 1 year later the animal was markedly obese, its body weight being 3.1 kg.

The sternal spot was found to be about two-thirds of its original size and was orange-yellow and dry instead of the usual moist dark brown. A heap of atrophic tissue represented the scrotum. An outline of a rudimentary pouch had, however, formed as two semilunar folds converging at the caudal end, though no actual recess was present. The collapsed scrotum was found to contain two bodies each about 0.3 cm. in diameter which were attached to the spermatic cords and were composed of remnants of epididymis and spermatic cord.

*Internal.* On opening the abdomen the viscera were found buried in an unusually large deposition of fat. The prostate was a thin long structure about 4 cm. long and maximally 0.5 cm. wide. The other organs in the abdominal cavity appeared to be normal.

On microscopic examination a section through the widest part of the prostate revealed extensive fibrosis, particularly around the urethra. The urethral lining was raised into numerous longitudinal folds, the usual circular appearance seen on cross-section having been lost owing to a degree of epithelial metaplasia on the one hand, and shrinkage of the urethral lumen on the other. The remaining vestiges of the prostatic tubules showed hyperplasia. A few tubules near the urethra contained necrotic debris and a few leucocytes. The periphery of the prostate consisted of a layer of fibrous tissue containing some muscular elements.

#### THE EFFECT OF CASTRATION AND ADMINISTRATION OF OESTROGEN

##### AND TESTOSTERONE PROPIONATE

#### *Animal and treatment*

This animal was discussed in a previous communication [Bolliger, 1944*a*]. After castration at the age of 6 months and the subsequent administration of 5.3 mg. of oestradiol dipropionate over a period of about 3½ months a complete transformation of the scrotum into a pouch took place. This pouch persisted for several months and then reverted to a scrotum. In February 1944, more than a year after the last injection of oestrogen, the pouch had practically disappeared, becoming evident only when the animal was struggling. The scrotum consisted of a thin tag of tissue about 2 cm. long. When lifted sideways it formed a triangle. The fur had a feminine appearance. The sternal spot consisted mainly of grey hairs with a smaller number of yellow-brownish hairs. It was about 3 cm. long and 1.5 cm. wide. The general health of the animal was good. It weighed 2.59 kg.

Injections of 20–25 mg. of testosterone propionate were started on 24 February and given every 2–4 weeks for 6 months. The animal was killed a year later.

*Details.* 20 mg. of testosterone propionate on 24 Feb. and 9 March 1944; 25 mg. on 23 March, 6 and 21 April, 5 and 19 May, 7 and 28 June, 19 July and 23 Aug. 1944. The animal was killed 27 Aug. 1945.

#### *Observations*

*External.* At the second injection the fur was already becoming yellow, and later (21 April) the sternal spot had become brown. This process continued with the

secretion of yellow-brown pigment (19 May), until after the final injection (23 Aug.) the whole animal was coloured a most extensive yellow-brown by a copious secretion of pigment which took place all over the body but was particularly marked over the sternal area which was a deep brown colour. The scrotum was about the same size as at the beginning of the experiment, and the pouch, which had been quite marked after the first few injections of testosterone propionate (Pl. 1, fig. 2), had diminished in size. However, about 2 months after stopping the injections of male sex hormone the animal began to lose its yellow colour and the dark brown sternal area became lighter. At the same time the animal, though originally rather stunted, began to reach very large dimensions and ultimately weighed 3.95 kg. The pelt was dark grey on the dorsum and resembled that of the female. The sternal patch was of slightly orange-brown colour interspersed with grey hairs. It measured 6.5 cm. in length and 2.5 cm. in width and was dry. The pouch was only faintly outlined by slightly raised lips and the scrotum was 1.9 cm. wide at its base and 2.0 cm. in length.

*Internal.* On opening the abdominal cavity the viscera were found to be embedded in large amounts of fat. The prostate was small; its maximum width was 1.5 cm. and its length 3.1 cm. It weighed 4.1 g. Faint pigmentation on its lower third was noticeable, particularly just below the muscular surface layer (Pl. 3, fig. 5).

On cross-section the urethra was branched. Stratified squamous epithelium had replaced the original columnar type. Comparatively large glandular tubules or cystic spaces surrounded by dense fibrous tissue, were observed adjacent to the urethra. Necrotic debris containing pus cells was noted in nearly all of these tubules. The original epithelial lining had been replaced by a stratified squamous variety. The surface layer of the prostate consisted mainly of fibrous tissue together with a number of muscle fibres (Pl. 4, fig. 8).

#### ADOLESCENT MALE: EFFECT OF CASTRATION, CHORIONIC GONADOTROPIN AND OESTROGEN

##### *Animal and treatment*

Injections of chorionic gonadotropin were given every 1–3 weeks to a young male possum starting when it was about 6 weeks old, and its mother had to be anaesthetized to allow the injections. The doses rose from 15 to 500 i.u. per injection. After 3½ months, when a total of 1720 i.u. had been given, the animal was castrated and a further 2500 i.u. given during 6 weeks. This was followed by a series of injections of oestradiol dipropionate (6.0 mg. during 8 months). The possum was killed 5 months after the last injection.

*Details.* Chorionic gonadotropin: 15 i.u. on 29 June and 10 July 1942, 20 i.u. on 22 July and 4 Aug., 50 i.u. on 19 and 27 Aug., 250 i.u. on 3 Sept. (wt. 120 g.), 400 i.u. on 14 and 28 Sept., 500 i.u. on 8 Oct. (wt. 250 g.), 3, 16 and 24 Nov. and 3 and 12 Dec.

Castration: performed on 16 Oct. (wt. 3 Nov. 520 g.).

Oestradiol dipropionate: 0.5 mg. on 31 Dec., 1.0 mg. on 12 and 25 Jan. and 8 and 23 Feb. 1943, 0.5 mg. on 28 May, 28 June and 5 Aug.

The animal killed: 1 Nov. (wt. 2.74 kg.).

### Observations

*External.* During the treatment with chorionic gonadotropin the scrotum developed and was already large and pendulous after 2 months' treatment, when the animal weighed 120 g. This development continued and was not interrupted by castration, while the injections of chorionic gonadotropin continued. The dimensions of the scrotum were  $4.0 \times 2.0$  cm. when the last injection of chorionic gonadotropin was given.

When the oestradiol dipropionate injections were started (31 Dec. 1942) the scrotum became oedematous and diminished to the size of a cherry, while definite pouch folds appeared. At this stage (23 Feb. 1943) the oestrogen injections had to be interrupted owing to urinary obstruction and the development of a huge bladder. A month later this condition had much improved and the pouch was well developed though the scrotal remnant persisted. The scrotum collapsed during the second bout of oestrogen treatment but was only partially involuted; it was never completely inverted at any stage. After stopping the oestrogen injections the scrotum reformed.

When the animal was killed 5 months later the dorsal fur was grey and there were none of the red-brown hairs in the scapular region which characterize a male of this age. The sternal area was of red-brown colour, the hairs were dry, and many of a light grey tinge; it was about 2.5 cm. long and 1 cm. wide. The scrotum was a structure measuring 2 cm. in width and 3 cm. in length, containing fat and the ends of the spermatic cords in the neck of the scrotum.

*Internal.* On opening the abdomen a considerable amount of intraperitoneal fat was found adhering to the organs, and the bladder was adhering to the abdominal wall in several places. The prostate (wt. 3.0 g.) was much diminished in size compared to that of a male of similar age; it measured 3 cm. in length and 1.1 cm. in maximum width.

On cross-section, the urethral wall exhibited numerous folds (Pl. 4, fig. 9). The usual columnar epithelium was replaced by a stratified squamous type. Cystic spaces were observed around the urethra. These spaces or tubular vestiges enclosed necrotic material containing leucocytes. Numerous leucocytes were also noted in the fibrous tissue surrounding the urethra. The outer layer consisted of muscular and fibrous elements, the former also occurring as fine strands within the prostate and near the urethra.

### DISCUSSION

In order to demonstrate beyond doubt the homology between scrotum and pouch, it had to be shown that the male scrotum could be converted into a marsupial pouch. In a previous paper this proof was furnished in the case of one specimen of *Trichosurus vulpecula*, though the internal changes were not studied [Bolliger, 1944a]. In the present experiment the scrotum was successfully converted into a pouch in another animal, and the second experiment was even more successful than the first one as the resulting artificial pouch was a symmetrical structure of complete permanency.

#### *Transformation of the scrotum into a marsupial pouch*

The experiments described in this paper in conjunction with others published previously [Bolliger, 1942c] establish the fact that castration followed by oestrogen administration in the adolescent male brings on a complete involution of the scrotum



and the formation of a marsupial pouch. This process was assisted by the additional administration of oestrogen before castration. Gonadectomy or oestrogen administration alone was unsuccessful. Castration *per se* brought on some scrotal contraction only and practically no infolding, while the administration of oestrogens, even in dangerously large doses, only produced a temporary testicular ascent, though frequently associated with the formation of a rudimentary pouch [Bolliger & Carrodus, 1939; Bolliger & Canny, 1941]. Castration and subsequent administration of oestrogen to a fully grown male also proved unsuccessful, indicating that age is a factor in the process.

Chorionic gonadotropin enlarges the scrotum in the male and the pouch in the female [Bolliger, 1942*a*], and when administered to a male before and after castration it only enlarges the empty scrotum. Subsequent administration of maximum doses of oestrogens produced a definite, though temporary, pouch, but complete inversion of the scrotum did not take place, as though the forces tending to enlarge the scrotum were still active and competing against the activity of the oestrogens.

In the experiment where a pouch had been created by castration and administration of oestrogen as previously described [Bolliger, 1944*a*] there was, however, a reformation of the scrotum some 4 months later and the question was examined as to whether the administration of large doses of male sex hormone (testosterone propionate) would still enlarge the atrophic empty scrotum. The result obtained was rather paradoxical, inasmuch as a pouch was recreated and the scrotum was largely, though not completely, inverted. This artificial pouch, however, began to disappear again while the animal was still being injected with testosterone propionate, and the final result was similar to that seen previously in this animal some 6 months or more after oestrogen administration, i.e. an atrophic small scrotum with traces of a rudimentary pouch. The somewhat unexpected appearance of a pouch after the administration of an androgen was in keeping with some earlier observations made on normal possums and their pouch reactions towards male sex hormone [Bolliger & Carrodus, 1940]. On that occasion even the pouch of the normal female was found to give what appeared to be a gynaecogenic reaction, and this was explained by the ambisexual properties of testosterone propionate where apparently the gynaecogenic or oestrogenic component acts very markedly on the pouch. This reaction, however, was also only temporary and the continuance of injections of testosterone propionate in the normal female, as in the castrated and oestrogen-treated male, soon led to an atrophic condition.

The transformation of the scrotum into a pouch definitely proves the homology of the two organs. Bresslau [1920] postulated the theory of marsupial pockets as the starting point of the pouch. It is difficult to reconcile this theory with the experimental findings on *Trichosurus vulpecula*, a diprotodont marsupial, as demonstrated in this paper.

#### *Changes in the prostate*

In these experiments, two distinct trends for the prostate are clearly discernible. Firstly, castration, in the adolescent animal, brings on practically complete abolition of the prostate gland. Secondly, the female sex hormones administered after castration definitely retarded this process and tended to produce an organ like that seen after the administration of oestrogens only, i.e. a small gland which, besides

extensive fibrosis, still contains numerous acini exhibiting marked epithelial changes, keratinization, and leucocytic infiltration. Paradoxically, the picture most reminiscent of oestrogen activity, was obtained after the administration of large amounts of testosterone following castration and oestrogen administration (p. 35 and Pl. 4, fig. 8). This again may be due to the gynaecogenic activity of testosterone propionate already mentioned in connexion with changes in the pouch. In the normal male, however, testosterone propionate produced marked glandular hypertrophy.

In all the experiments described the lumen of the prostatic urethra changed markedly in shape and size. The wide (2–4 mm.) round urethra with a smooth unbranched interior seen in the normal animal, became narrower and developed numerous longitudinal folds. The total size of this changed urethra was much smaller than in normal animals. For example, the entire prostatic remnant obtained after castration could almost be placed in the lumen of the normal prostatic urethra. Consequently on cross-section the area covered by the branched lumen of the prostatic urethra from the castrated animal was only about a tenth of its original size as judged from histological preparations. The epithelium also undergoes modification and thus the picture of the prostatic urethra in the castrated male, with or without further hormone treatment, resembled that of the urethra in higher mammals. It is noteworthy, however, that this change of shape and size of the urethra in a castrated male without further hormone treatment, produces no ill-effects such as urinary obstruction and it is believed that on the evidence on hand the wide lumen of the prostatic urethra of the normal male is a sex characteristic which may only be necessary for the process of reproduction. The copious secretion from the exceptionally large marsupialian prostate may require a wide urethra in order to allow satisfactory mixing with the accumulation of spermatozoa present [Bolliger, 1942*b*]. It seems, therefore, quite possible that the wide prostatic urethra performs, to a certain extent, the functions of seminal vesicles and ampulla, organs which are absent in marsupials.

#### *Changes in pigmentation*

In previous communications it has been pointed out that the pelage of the male *Trichosurus vulpecula* shows distinct differences from that of the female [Bolliger & Hardy, 1944]. On the dorsum the male is characterized by brown hairs which are particularly numerous in the dorsal thoracic region. In addition, on the sternum the male possesses a typical spot of dark brown oily hair which is present in the female in a much smaller degree. Internally the male exhibits a grey pigmentation covering the surface as well as the substance of the lower half of the prostate gland. The sternal and dorsal characteristics are under the control of sex hormones [Bolliger, 1944*b*]. This has been confirmed in the present experiments and, in addition, it was also shown that the pigmentation of the prostate depended on the same influences as the sternal hairs because the castrated animal which had no pigmentation on the sternal pelage also had no pigmentation of the prostatic remnant. Those animals which were castrated and given oestrogens developed a sternal spot comparable to that seen in females castrated after reaching puberty, while the prostate was devoid of any pigmentation.

On the other hand, testosterone was found to restore the characteristic sternal spot of the male in castrated males (p. 35). It is also known from previous experi-

ments that the pigmentation of the normal prostate is greatly increased by the administration of testosterone propionate. Therefore it may be assumed that, in the animal described, considerable pigmentation of the prostate developed after the administration of the male sex hormone. However, at the time when the animal was killed, that is a year after terminating the injections, a faint but definite pigmentation was observed on the lower part of the small prostate. This was assumed to be a remnant of the intense pigmentation produced immediately after the first few injections of testosterone propionate. On the other hand, the pigmentation of the sternal as well as dorsal hairs observed after the administration of testosterone propionate had practically disappeared at the time the animal was killed.

In connexion with these observations, reference is made to a recent paper by Finkel [1945] on the relation of sex hormones to pigmentation in the opossum (*Didelphys Virginiana*) and ground squirrel.

#### General

Besides changes in pouch, prostate, and pigmentation certain other features common to the experiments described are worth mentioning. In contrast to similar investigations described by American authors [Burns, 1939; Moore, 1941] on the pouch young of *Didelphys Virginiana* the animals dealt with in the present study are adolescent ones which during the process of the experiment reach the age of maturity. Notwithstanding the great difference in age the changes observed are almost as dramatic as those seen in pouch young. To our knowledge this is typical of the diprotodont marsupial because polyprotodonts such as *D. Virginiana* in the hands of the American authors or *Parameles nosatu* in our hands, have, so far, not shown similar responsiveness. It is also remarkable how sensitive these animals are to small doses of oestrogens when compared, for example, with rodents of the type ordinarily used in laboratory work. In the experiments described in this paper a few milligrams were sufficient to convert the scrotum into a pouch, while 10 mg. or more might not be sufficient to produce any macroscopic response in a rodent of a similar size. Doses of oestrogens of about 4–6 mg. administered to normal or castrated males during a few weeks bring on serious urinary retention and frequently death. However, some slight degree of tolerance can be produced by beginning with small doses and working up to larger ones, as demonstrated in the experimental part of this paper.

Finally, it may be stated that in accordance with many other observations, the propionic acid ester of oestradiol produces an essentially similar response to the synthetic oestrogen stilboestrol.

#### SUMMARY

The administration of oestrogen to an adolescent castrated male marsupial (*Trichosurus vulpecula*) brought on a complete transformation of the scrotum into a pouch, thus confirming a previous experiment. The pouch formed was permanent.

Castration *per se*, or the administration of oestrogen to a fully grown castrate, did not change the scrotum into a pouch.

The administration of chorionic gonadotropin to an adolescent castrate before and after operation hindered the complete transformation of the scrotum into a pouch.

Testosterone propionate was administered to a gonadectomized male in which complete transformation of the scrotum had been achieved. Paradoxically, a transformation into a temporary pouch resulted.

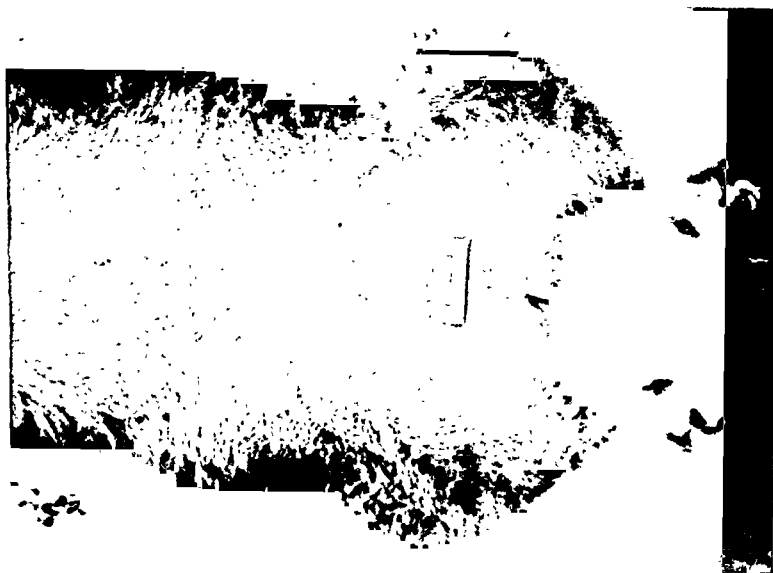


FIG. 1. Empty scrotum 11 months after castration. The scrotum, though small, is persistent and no pouch has formed (see p. 32 and figs. 3 and 6).



FIG. 2. Marked pouch formed after the administration of oestrogen and testosterone propionate to a castrated male (see p. 36 and figs. 5 and 8). Note the serotal remnant within the pouch. The abdominal hairs have been clipped.

External appearance of scrotal area of treated animals      Comparable normals are depicted by Bolliger & Canny [1941].



FIG. 4. Effect of oestrogen treatment in adolescent, castrated animal (p. 34 and fig. 7), whose scrotum became completely inverted to a pouch. Note the absence of prostatic pigmentation.

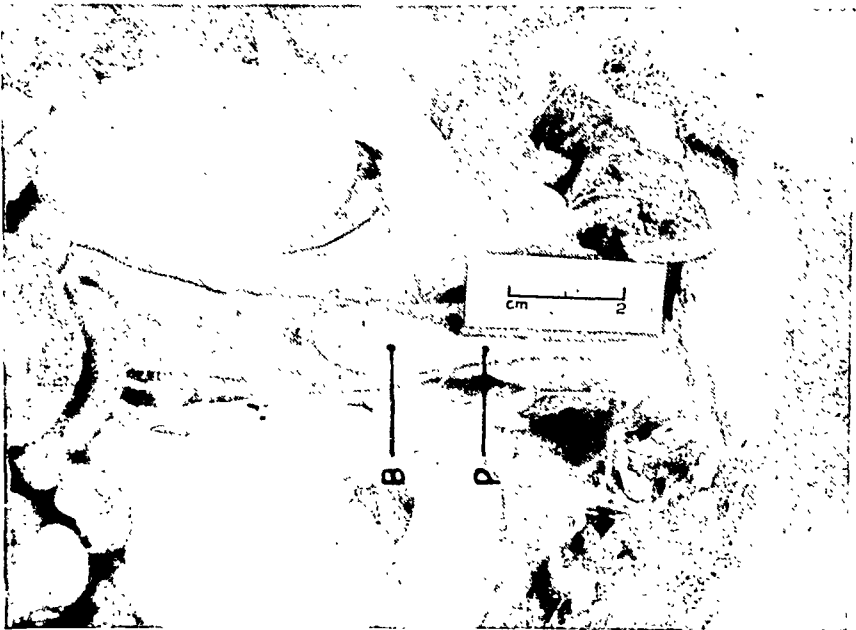


FIG. 3. Prostate remnant in male 11 months after castration (p. 32 and figs. 1 and 6). The fat covering and adhering to prostate and bladder has been removed though small globules still adhere to the narrow prostate. B = bladder, P = prostatic remnant.



FIG. 5. Prostate and bladder as well as large amounts of peritoneal fat in a castrated animal treated with oestrogen and testosterone propionate (p. 36 and figs. 2 and 8).



FIG. 6. Cross-section through the widest portion near the cephalic end in an 11-months castrated male. Note the thick outer covering, severely branched urethra and absence of glandular tissue (p. 33 and figs. 1 and 3).

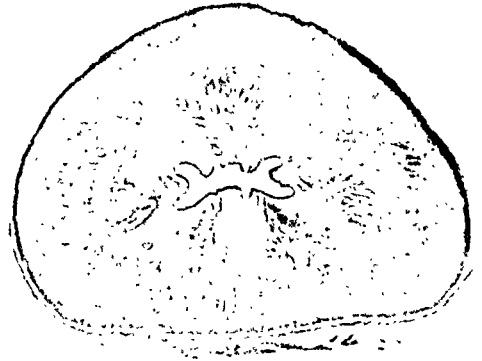


FIG. 7. Branched urethra and vestiges of glandular tubules in a castrated adolescent male treated with oestrogen (p. 34 and fig. 4).

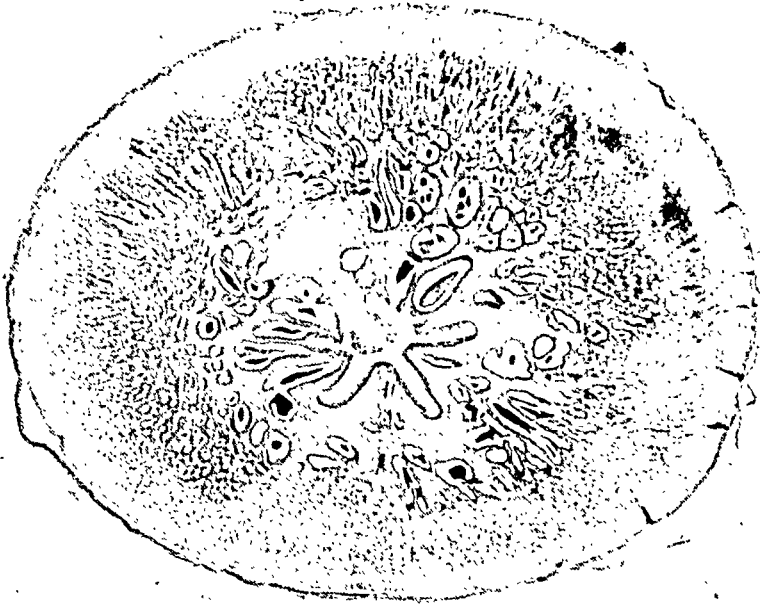


FIG. 8. Section through widest part of prostate in castrated male treated with oestrogen and testosterone (p. 36 and fig. 5). Note the branched urethral lumen and numerous cystic spaces, many containing keratin plugs.



FIG. 9. Cross-section below the widest part in castrated animal treated with gonadotropin and oestrogens (p. 37). Note branched urethral lumen and cystic spaces, some plugged with keratin.

These treatments have a marked influence on the fur of the animal, as is probably best shown by the sex differences in the sternal area, which disappeared completely after castration. Subsequent oestrogen administration produced a sternal area akin to the female type and testosterone reconverted it to an area of markedly male character.

The prostate, which after castration underwent complete atrophy, was of somewhat larger, though still very small, size in castrates which were also given oestrogen. Additional administration of testosterone further increased the size of the atrophic prostate.

In all the experiments described, including castration *per se*, the prostatic urethra, originally a wide, slightly conically shaped tube, contracted and became a narrow branched channel lined by squamous epithelium.

Castration with or without the administration of oestrogen abolished the pigmentation of the prostate.

One of us (A.J.T.) had a research grant from the National Health and Medical Research Council, Commonwealth of Australia.

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# THE POSSIBILITY OF DETERMINING ANDROGEN PRODUCTION BY MEASURING THE ACID PHOSPHATASE IN SEMEN: INVESTIGATIONS IN CRYPTORCHID PATIENTS

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The determination of the androgen excretion in the urine has, in spite of the disadvantages, been the method of choice in studies of the androgen production in the organism.

The presence of a specific acid phosphatase in prostatic tissue [Kutscher & Wolbergs, 1935] suggested that a relation might exist between the androgen production and the amount of phosphatase in semen. Gutman & Gutman [1939], by injection of testosterone propionate in prepubertal rhesus monkeys, produced a several-fold increase of phosphatase.

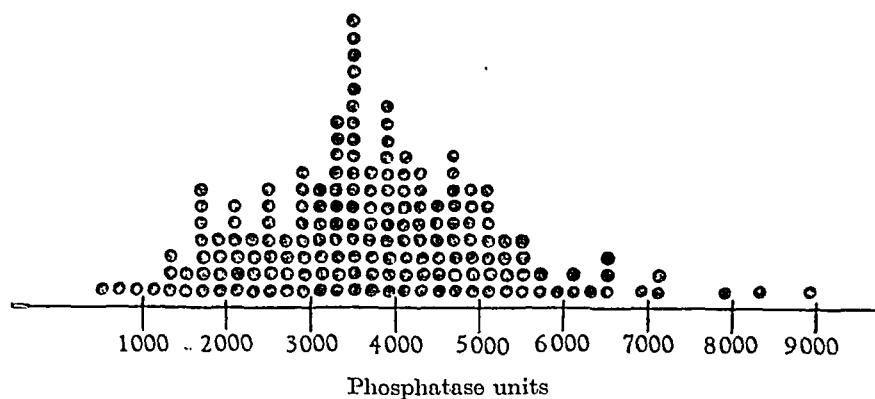


FIG. 1. Amount of phosphatase found in semen of 173 normal men.

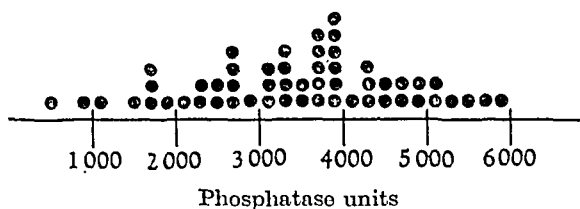


FIG. 2. Amount of phosphatase found in semen of 54 patients suffering from aspermia.

## NORMAL STANDARDS

The amount of phosphatase in the semen of normal men varies greatly, but in each individual is relatively constant [Gutman & Gutman, 1940]. In unpublished investigations Sury found an average of 4400 phosphatase units per ml. in 50 normal

individuals, and Andersson an average of 3700 units per ml. in 173 normal individuals. In aspermia the two investigators found 3300 and 3400 units respectively. The method employed for determining the amount of acid phosphatase in semen has been the same as used by Gutman & Gutman [1939]: a unit being that degree of activity which at 37° C. will liberate 1 mg. of phenol in 1 hr. from the specified monophenyl-phosphate substrate-citrate buffer solution at pH 4.9.

The investigations by Andersson on the amount of prostatic phosphatase in the semen of normal men and men suffering from aspermia were made in 173 and 54 individuals respectively. This material has been used for comparison with the amounts found in the present investigation. The results of the phosphatase analysis of the 173 normal men and the 54 men suffering from aspermia are given above in Figs. 1 and 2.

#### EXPERIMENTAL

The purpose of this work has been to investigate the correlation between the amount of acid phosphatase in the semen and the androgen excretion, and also to study the effect of various exogenous hormones upon the amount of phosphatase in the semen.

To fulfil this purpose the persons examined had to be a uniform group in which a certain degree of testis' insufficiency was present. One of us [Engberg, unpublished] in examining bilateral cryptorchids has found the androgen output to be lowered, the androgen excretion in 44 normal men averaging 49 i.u. and in 38 adult bilateral cryptorchids 26 i.u.

For this investigation we have chosen as material bilateral cryptorchids between 17 and 41 years of age without signs of prostatic inflammations. None of them has suffered from gonorrhea. In all patients aspermia has been found.

The excretion of androgens in urine has been measured by the capon comb method [Hamburger, Halvorsen & Pedersen, 1945]. The amount of phosphatase in semen has been determined by the method of Gutman & Gutman [1939]. The androgen excretion has in several cases been determined 1-2 years before the determination of acid phosphatase.

The determination of androgen excretion for one of the patients (no. 3) has been found to be incorrect, the received 24 hr. sample of urine being incomplete. The result in this case has been excluded from the statistical considerations. In two patients (nos. 8 and 15) very low amounts of acid phosphatase were found. Contrary to the others, these patients were at the moment of the investigation very hard working, both during the day and in the evening. Those cases have been included in the statistics.

The results of the determination of androgen in the urine and of the phosphatase in the semen are given in Table 1 and graphed in Fig. 3.

In Table 2 the results of treatment of cryptorchid patients with hormones are given. The amount of acid phosphatase in semen has been determined the day before and the day after treatment.

#### DISCUSSION

The investigations presented seem to prove that—at least in cryptorchids—a certain correlation exists between the androgen production of the organism determined by the androgen excretion in the urine and the amount of acid phosphatase in the semen. This in spite of the uncertainty in both methods of determination, for the method of

Table 1. *Results of urine analyses for androgenic hormone and determination of acid phosphatase in semen*

Pt. no.	1	2	3	4	5	6	7	8	
Age	40	17	23	26	31	30	23	32	
Androgen (in units)	4	13	15	20	20	25	25	29	
Phosphatase units	750	1310	3750	1989	1490	1500	3485	945	
Pt. no.	9	10	11	12	13	14	15	16	17
Age	41	26	21	34	29	25	26	32	23
Androgen (in units)	30	30	32	40	40	42	45	45	45
Phosphatase units	1790	1800	1800	2650	3900	2550	790	3230	3000

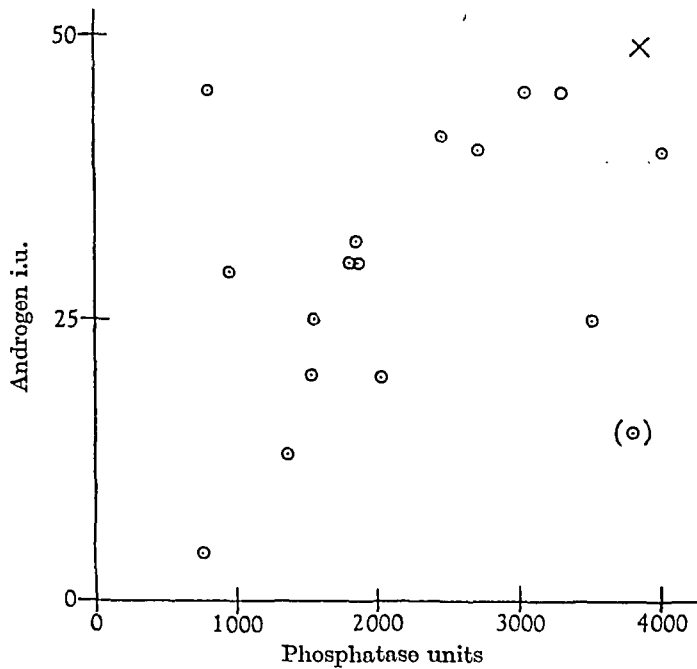


FIG. 3. The results of Table 1 presented graphically. The two crosses mark the average amount of phosphatase in semen, as found by Andersson and Sury and are placed at the level of 49 i.u. of androgen, which is the average amount excreted in 24 hr. by the 44 normal men.

Table 2. *Variation in semen phosphatase after hormone treatment of cryptorchid patients*

Dosage and preparation used	Patient	Androgen in urine (i.u.)	Phos- phatase before injection	Phos- phatase after injection	Difference	
					Total	%
Gonadotropic hormone treatment						
P.U. extract (Physex) 3000 i.u. daily for 10 days	4	20	1989	3839	+ 1850	+ 93
	8	29	945	2677	+ 1732	+ 183
P.M.S. extract (Antex) 3000 i.u. daily for 10 days	1	4	750	2700	+ 1950	+ 260
	5	20	1490	1950	+ 460	+ 31
	2	13	1310	1400	+ 90	+ 7
Androgen treatment						
Testosterone propionate (Per- andren) 10 mg. daily for 10 days	1	4	650	1100	+ 450	+ 69
	13	40	3900	2145	- 1755	- 45
	14	45	790	2105	+ 1315	+ 166
Oestrogen treatment						
4:4' diacetoxy- $\gamma\delta$ -diphenyl- $\beta\delta$ - hexadiene (Klianyl) 8 mg. daily for 10 days	12	40	2505	780	- 1725	- 69
	10	30	2635	3825	+ 1190	+ 45
	11	32	2123	5355	+ 3232	+ 152

androgen analysis is of uncertain accuracy while the semen phosphatase determination must depend on the proportion of prostatic secretion in the semen. Concerning this last item the possibility of mistakes will probably prove greater in normal individuals than in cryptorchids, in whom the part of the semen from the testes is absent.

If simultaneous investigations of urinary androgen and of the acid phosphatase in the semen, showed the same correlation in normal individuals as are here shown in cryptorchids, it would be possible to get an impression of the androgen production in the organism by measuring the acid phosphatase in semen alone.

The variations in semen acid phosphatase after the application of various hormones seem to indicate that the applied hormone has some action on the production of acid phosphatase, though the limited number of observations do not permit any conclusions at present. The action of oestrogen in this respect is remarkable as previous investigations have not attributed to the oestrogens any special effect on the epithelial tissue of the prostate gland.

#### SUMMARY

In adult bilateral cryptorchids a correlation between the androgen excretion in the urine and the amount of acid phosphatase in the semen has been demonstrated. The possibility of determining acid phosphatase as a measure for the formation of male sexual hormone is suggested. The application of exogenous hormones seems to alter the amount of phosphatase, and in two cases an increase in the amount of semen acid phosphatase has been found after the administration of oestrogen.

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#### ADDENDUM: MATHEMATICAL-STATISTICAL COMMENTS

By T. BUSK

In the preceding paper some observations on the amount of acid phosphatase in semen from normal men and men suffering from aspermia are given. The frequency distributions of these amounts are shown in Figs. 1 and 2. Similarly the amounts of phosphatase in semen from a number of cryptorchid men are given in Table 1.

In order to analyse these distributions the following method (the 'probit' method) has been applied. For all the amounts  $x = 600, 800, 1000, \dots$  (acid phosphatase units) the relative number  $y$  of men with an amount of acid phosphatase equal to or less than  $x$  has been observed. The corresponding values  $(x, y)$  are plotted in the 'probit' diagram in Fig. 4. The scale for the abscissa is an ordinary metric one, but the scale for the ordinate is of such a nature that a sample, which is normally distributed, always will be presented approximately as a straight line.

From Fig. 4 it may be seen that the sample comprising normal men is mainly in agreement with the normal distribution, but a departure is found for the greatest values, which show too high a frequency in the sample. It seems possible that the

observed population has to be regarded as an inhomogeneous one consisting of two fractions, one of these containing men with a very high amount of acid phosphatase.

Treating the observations on the amounts of acid phosphatase in semen from 54 aspermic men the other probit diagram given in Fig. 4 has been found. It is seen that in this case a very good approximation to a straight line has been found and consequently there is a good agreement with the normal frequency distribution.

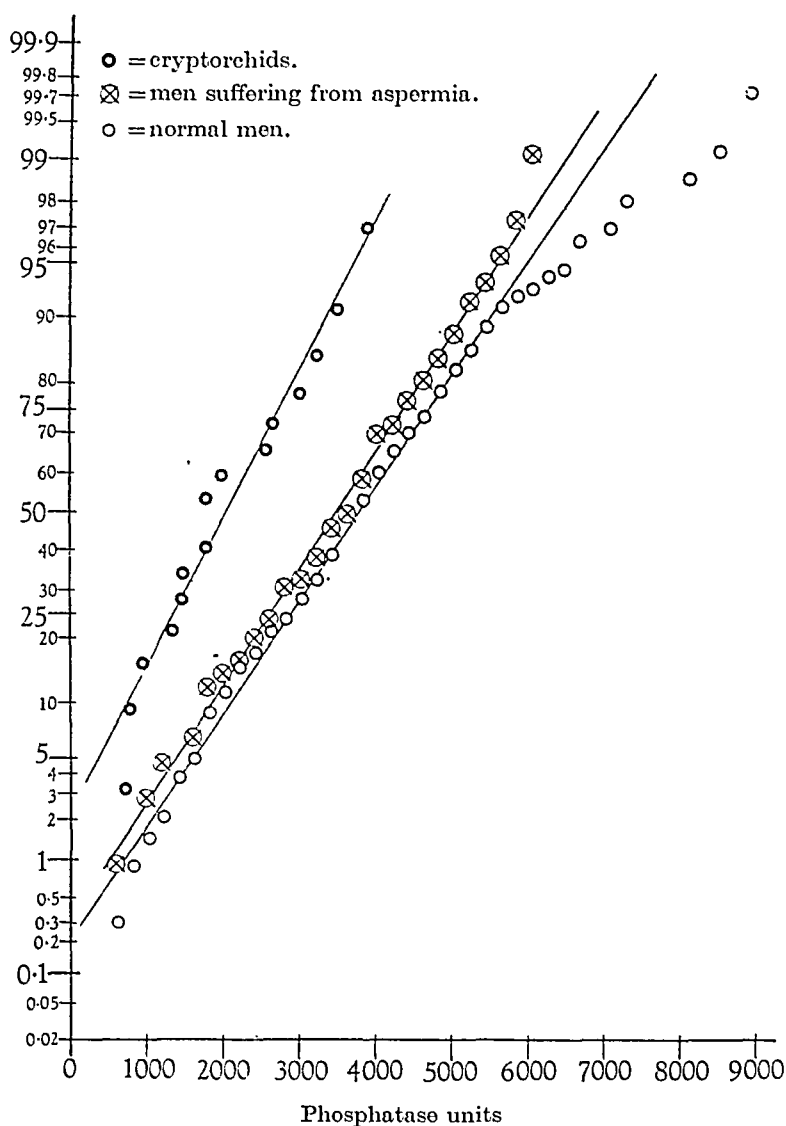


FIG. 4

The observations on the amounts of acid phosphatase in semen from 16 cryptorchid men form the third probit diagram in Fig. 4. If the smallness of this sample is taken into consideration agreement with the normal frequency distribution is found also here.

Table 3 contains the results of the calculations of the mean and the standard deviation of the samples mentioned above.

Table 3. *Mean values and standard deviations in the three groups*

Patients	No. of observations	Amount of acid phosphatase	
		Mean	Standard deviation <i>s</i>
Normal men	173	3767	1451
Aspermic men	54	3434	1254
Cryptorchid men	16	2061	974

From this table it is seen that the standard deviation of the amount of acid phosphatase is less for aspermic, than for normal, men and less for cryptorchid, than for aspermic, men. In order to test whether these differences are significant R. A. Fisher's *z*-test has been applied and the ratio  $e^{2z} = s_1^2 : s_2^2$  and the 5 % level of this ratio for the corresponding degrees of freedom taken from Fisher and Yates's tables are given in Table 4.

Table 4. *Statistical comparisons between groups*

Comparison between	<i>z</i> -test			<i>t</i> -test			
	$e^{2z} = s_1^2 : s_2^2$	Degrees of freedom	5 % level of $e^{2z}$	<i>t</i>	Degrees of freedom	10 % level of <i>t</i>	0.1 % level of <i>t</i>
Normal and aspermic	1.339	(172.53)	1.47	1.52	225	1.65	—
Aspermic and cryptorchid	1.656	(53.15)	2.17	4.03	68	—	3.44
Normal and cryptorchid	2.218	(173.15)	2.10	4.60	187	—	3.35

From this it is seen that the differences between the standard deviations of the amount of acid phosphatase in semen from normal and aspermic and from aspermic and cryptorchid men cannot be regarded as significant. Nor does it seem possible to regard the difference between the standard deviations for normal and cryptorchid men as significant if the departure from the normal frequency distribution found for the acid phosphatase amount for normal men is taken into account.

From Table 3 it is also seen that the mean amount of acid phosphatase is less for aspermic than for normal men and also less for cryptorchid than for aspermic men. Applying 'Student's' *t*-test the values in Table 4 have been computed and from these it is seen that with regard to the mean amount of acid phosphatase there is a significant difference between cryptorchid and normal men and between cryptorchid and aspermic men but not between normal and aspermic men.

A last computation has been made in order to test whether in cryptorchid men the amount of acid phosphatase in semen is correlated with the amount of androgenic hormone. In the preceding paper it is mentioned that the observation of androgenic hormone for patient no. 3 is incorrect and this observation is therefore omitted from the calculations.

The method applied to test the significance of the correlation is that given by R. A. Fisher and his table has been used for the calculations. The condition that a correlation coefficient is not meaningless is that the distribution corresponds to a normal correlation surface. That this condition is fulfilled has been tested by drawing

probit diagrams for each of the variables and the accordance has been found to be good. (Here it may also be remembered that a normal frequency distribution has been found for the amount of acid phosphatase in normal and aspermic men.)

By the calculations a value of  $r = 0.5168$  has been found for the correlation coefficient and the 5 and 2 % levels of  $r$  given by Fisher's table are 0.4973 and 0.5742 respectively. From this it is seen that a correlation coefficient greater than the observed will only be found in 4.2 % of samples of *uncorrelated* variables, and the correlation can therefore be regarded as significant. Furthermore this result is supported by the circumstance that by an omission in the calculations of patients nos. 8 and 15 (which perhaps is justified by their special circumstances) the highly significant value  $r = 0.7529$  is found.

# THE ANTIGENIC PROPERTIES OF IODINATED PROTEINS

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Detailed precipitin studies have been made of the antigenic properties of thyroglobulin, but some of the results have been criticized on the grounds of lack of purity of the thyroglobulin employed [Hektoen & Schulhof, 1923; Lerman, 1940; Stokinger & Heidelberger, 1937]. The physiological investigation of antithyroglobulin sera, which might throw light on the problem, has been much less pursued. The early results of Rogers & Beebe [1908] suggested that animals could be passively immunized against exogenous thyroglobulin, but several later investigators failed to obtain positive results [Schulhof, 1930; Rosen & Marine, 1937]. Recently, however, Lerman [1941, 1942] has claimed that antithyroglobulin sera will not only immunize against exogenous thyroglobulin, but will also inhibit the action of endogenous thyroglobulin and ultimately lead to a condition of myxoedema. The substance is organ specific rather than species specific, so that antiserum to thyroglobulin obtained from one species will inhibit the activity of that from another species.

The work of Clutton, Harington & Yuill [1938] is relevant in this connexion. These authors prepared artificial antigens by introducing thyroxine into the albumin or globulin molecule and additional thyroxine into thyroglobulin, and investigated their immunological and physiological properties. Antiserum against thyroxyl globulin gave a strong precipitin reaction with the homologous antigen and with thyroxyl thyroglobulin, a fairly strong one with thyroxyl albumin, a weak one with thyroglobulin and none with globulin. Antiserum against thyroxyl thyroglobulin behaved similarly, except that the reaction with thyroxyl albumin was weak. Antiserum against thyroxyl albumin gave a strong reaction with its own antigen, a less strong one with thyroxyl thyroglobulin and thyroxyl globulin, a weak one with thyroglobulin and none with albumin. In experiments on the basal metabolic rate of rats, Clutton *et al.* [1938] found that injection of antiserum to thyroxyl globulin and thyroxyl thyroglobulin protected the animals from the effects of exogenous thyroglobulin. They failed, however, to find that treatment with the antisera had an effect on the basal metabolic rate of normal rats, i.e. that the antisera interfered with endogenous thyroidal activity.

The observations on thyroglobulin and thyroxyl proteins raised the question of the possible antigenic properties of another group of substances with thyroidal activity, i.e. those produced by the artificial iodination of various proteins having a high tyrosine content [see Pitt Rivers & Randall, 1945]. Preparations with good biological activity can now be produced; iodinated casein and iodinated Ardein\* appear to be the most promising. These substances are fully active by mouth, but for many laboratory purposes they are more easily administered by injection, so that the question

\* Ardein is the registered trade mark for the ground-nut protein produced by I.C.I. (Explosives) Ltd.



of whether an animal would be immunized against them when so administered is of some importance. Rowlands [1945] failed to find that thyroidectomized rats chronically injected with iodinated Ardein became insensitive to its growth-promoting properties, and he concluded, therefore, that these animals were not actively immunized against this substance. However, it still seemed likely, from the work of Haurowitz & Appel [1939], that iodinated Ardein and other iodinated proteins would, under suitable conditions, evoke the formation of antibodies which might interfere with their biological effectiveness. The experimental demonstration of such antibodies, however, did not promise to be easy. At present, there is no simple quantitative method for assaying iodinated proteins or thyroid preparations on laboratory mammals, or, by contrast, for assaying substances inhibitory thereto. It seemed worth while, therefore, to consider whether the *Xenopus* tadpole technique described by Deanesly & Parkes [1945] for the assay of iodinated proteins and other substances having thyroïdal activity could be adapted to the assay of antisera. Owing to the small size of the tadpole, injection is impracticable, and the substances have to be added to the medium in which the tadpoles are maintained. The precise method whereby, under these conditions, the tadpoles take in the thyroid preparations and the iodinated proteins is not clear. These substances, however, are active by mouth and are probably eaten from suspension or drunk in solution by the tadpoles. Whatever the method, there is no doubt that the substances are highly effective when added to the medium. The technique, however, presents obvious difficulties when applied to the assay of antisera, which are generally assumed to be inactivated in the digestive tract. The whole orientation of the present research has been determined by the peculiarities of the tadpole technique.

In the first experiments, the antiserum was added to the medium containing tadpoles, either before or simultaneously with the iodinated protein. As with the anti-tadpole sera already described [Parkes, 1946*a*], these preparations became very turbid in the course of a day or two, and few tadpoles survived the period of treatment. Little progress was made with experiments of this type until the technique was evolved of adding sodium sulphathiazole to the medium to inhibit bacterial growth. In the meantime, it had been found that a vigorous precipitin reaction took place between the iodinated protein and the antiserum, and that the resulting precipitate, separated as completely as possible from the supernatant fluid, showed thyroïdal activity when tested on tadpoles by the usual technique. This led to a considerable amount of work to determine optimal proportions and to assay the thyroïdal activity of precipitates produced on a fairly large scale. In the account which follows, no attempt has been made to keep to the chronological order of the work.

#### MATERIAL AND TECHNIQUE

*Source and activity of iodinated proteins.* The following iodinated proteins were used.

Iodinated ox plasma	N4
Iodinated ox plasma	91973
Iodinated Ardein	N4 SF
Iodinated Ardein	DT/S/834
Iodinated casein	CB 1 + 2

The preparation of these substances, with the exception of the second batch of plasma material, has been fully described by Pitt Rivers & Randall [1945]. Iodinated plasma 91973 was prepared by Boots Pure Drug Co. Ltd., by the same technique as that used for the iodinated casein.

The activities and iodine contents of these preparations, relative to that of the laboratory standard, iodinated casein NC4+5, are given in Table 1.

Table 1. *Biological activity and iodine content of iodinated proteins and thyroid preparations*

Preparation	Iodine content (%)		Activity relative to standard
	Total	Acid-insoluble	
Iodinated casein NC4+5	8.1	1.6	1.0
Iodinated ox plasma N4	5.4	0.4	0.11
Iodinated Ardein N4 SF	3.61	0.5	0.29
Iodinated plasma 91973	6.52	0.54	0.26
Iodinated Ardein DT/S/834	7.6	1.6	1.43
Iodinated casein CBI+2	7.74	1.64	0.89
Thyroid 628423	0.27	0.10	1.0
Sheep thyroid 3	0.44	0.09	1.38
Horse thyroid 3	0.49	0.10	1.45
Ox thyroid 2	0.68	0.11	1.43
Pig thyroid 2	0.59	0.11	1.86

The biological values were obtained in the *Xenopus* tadpole test described by Deanesly & Parkes [1945] and are mainly taken from the earlier publication. The assay of iodinated plasma 91973 is new, and that of iodinated Ardein DT/S/834 augmented from the results previously recorded. The iodine analysis of iodinated plasma 91973 was carried out by Boots Pure Drug Co. Ltd., and that of DT/S/834 by I.C.I. (Explosives) Ltd. The other analyses are taken from Pitt Rivers & Randall [1945].

The iodinated proteins were administered to rabbits and tadpoles as suspensions in saline and water respectively. For precipitation tests, the solutions were prepared as follows: 50 mg. of dry substance was dampened with water and incubated in 3 ml. of *N*/10 NaOH until dissolved. About 45 ml. of distilled water was then added, and the solution was brought to approximately pH 7.4 by the addition of *N*/10 HCl. The volume was brought up to 50 ml. with distilled water, giving 1 mg. of the active substance per ml.

*Thyroid preparations.* Four preparations of thyroid gland, listed in Table 1, were used. The source of these preparations has recently been described [Parkes, 1946*b*]. They were crude extracts of the glands of different species and were fat and fibre free, and consisted mainly of denatured thyroglobulin. Their activities, relative to that of thyroid 628423, a commercial preparation used as the laboratory standard, and their iodine contents, are shown in Table 1. The values are all taken from a recent paper [Parkes, 1946*b*]. Solutions and suspensions of the thyroid preparations were made by the same method as that described above for the iodinated proteins.

*Preparation of antisera.* Rabbits were injected subcutaneously for 6 days a week, with 50 mg. per day of the active substance, for periods up to 3 months, a routine similar to that which would be used in treatment for physiological purposes. In most cases, weekly blood samples were taken, the first before injection, until the rabbits were bled out at the end of the course of treatment. The serum was obtained by direct

clotting of the blood, and was Seitz-filtered and cold-stored or freeze-dried until use. Serum was also obtained from a sheep chronically injected with iodinated casein (1 g. daily).

*Precipitin tests.* Small-scale *in vitro* tests of the reaction between antigen and antisera were made by precipitin tests, with a view to determining suitable proportions of antigen and antiserum for the large-scale preparation of precipitate to be tested on tadpoles. In the large-scale work it was desirable to restrict the total volume as much as possible, and in the small-scale tests the antigen and antiserum were mixed in different amounts of the same concentration rather than in the same amounts of different concentrations. These tests were carried out by mixing in small tubes a constant amount of the antigen solution, usually 0.05–0.25 ml. (0.05–0.25 mg.) with graded amounts of the antisera, 0.05–2.5 ml. This technique meant that, in any one series, the volumes in the tubes differed greatly, but tests on parallel series in which the volumes were made up to a constant level with saline, showed that, other things being equal, this inequality had little effect. The tubes were incubated at 37° C., observation being kept for flocculation as detected with the aid of a hand lens. In many instances, flocculation became visible in 10–30 min. At least 12 hr. were allowed to elapse before a lack of reaction was finally recorded. The optimal proportions of antigen and antiserum, as judged by speed of reaction, did not necessarily seem to give the maximal amount of precipitate. In the small-scale tests, however, it was very difficult to estimate closely the amounts of precipitate obtained, and the quantity of serum available permitted only a few large-scale experiments with varying proportions of antigen and antiserum. Other large-scale precipitations were therefore carried out with the optimal proportions of antigen and antiserum as determined by the speed of reaction in the small-scale precipitin tests.

*Preparation of precipitates for biological assay.* The antisera, in amounts up to 100 ml., were mixed with the antigens in the desired proportions in 100 ml. centrifuge tubes, which were incubated at 37° C. for suitable periods. The precipitates were centrifuged down, separated from the supernatant fluid, dried in acetone and ether, and weighed. Where the optimal proportions of antigen and antiserum were used, the amount of the precipitate was usually one to three times that of the antigen involved, but since the precipitates were not washed it is difficult to know how much of the material was non-specific. It was considered unwise to attempt to wash the precipitates before drying. The amount of supernatant fluid unavoidably left with the precipitate could have contained only a negligible amount of active substance. The supernatants were discarded, since it was not possible to carry out biological assay of the residual active material by the tadpole technique (see p. 56).

*Biological assay.* The substances to be tested for biological activity were assayed by the *Xenopus* tadpole method described by Deanesly & Parkes [1945]. The technique consists essentially of using *Xenopus* tadpoles about 20 mm. long (3 weeks old). A group of five is put into a 250 ml. beaker with 200 ml. of water containing the material to be assayed for thyroïdal activity. The beaker is kept at 26–27° C., and after 3 days the tadpoles are changed to clean water. The result (proportion of tadpoles with front legs erupted) is recorded on the 7th day from the beginning of the test.

## PRECIPITIN REACTIONS

At least one batch of antiserum was prepared against each of the nine preparations (excluding the two standards) mentioned on p. 51. Preliminary tests showed that definite precipitin reactions could be demonstrated with these sera and a systematic examination was therefore made of most of the eighty-one possible combinations of antigen and antiserum. In addition, appropriate antisera were tested against solutions of Ardein and casein, and against ox, sheep and horse serum. Various control tests, all of which were negative, were also carried out with normal rabbit serum. For all this work the antigens were made up at 1 mg./ml., and in only a few instances, even with homologous combinations, was the optimal amount of antiserum less than 1 ml./ml. of antigen. The antisera cannot, therefore, be said to have been very potent. This result was due probably to the method of immunization or to the active substances being poor antigens, and may have been contributed to by the routine Seitz filtration of the sera. The results are summarized below according to the nature of the combination.

*Antisera to iodinated proteins with iodinated proteins.* Antisera to each of the five iodinated proteins were tested against each substance. A precipitin reaction was obtained with all of the twenty-five possible combinations, but the vigour of the reaction varied greatly. In general, all the antisera reacted most vigorously with the homologous antigens or with those of similar type. Thus antisera to the two different iodinated plasmas reacted vigorously with either antigen, and the same applied to the two preparations of iodinated Ardein. These homologous reactions seemed to have no relation to the biological activity or iodine content of the antigens, and were undoubtedly complicated by the normal reactivity of the protein. The vigour of the heterologous reactions, by contrast, appeared to be correlated with the biological activity of the antigens used to produce the antisera, which in turn, in this series of preparations, was correlated with the acid insoluble iodine content. Thus, the antisera to iodinated plasma N4, which had only slight biological activity, reacted very feebly with the heterologous antigens. Antisera to iodinated plasma 91973 and iodinated Ardein N4 SF, two preparations of somewhat greater but still low biological activity, reacted more strongly with the heterologous antigens. Antisera to iodinated Ardein DT/S/834, the most biologically active substance of the series, reacted strongly with iodinated casein CB1+2, which also had good biological activity, and less strongly, though still markedly, with the less active antigens. Iodinated Ardein DT/S/834 was undoubtedly the best antigen of the series, and it was noticeable that antiserum to iodinated casein CB1+2 reacted at least as vigorously with the DT/S/834, as with the homologous preparation. By contrast, the heterologous reactions of iodinated Ardein N4 SF were noticeably feeble, an observation that may be connected with the fact that this material was salted out from the mother liquors after the main bulk (N4MB) of the iodinated Ardein had been obtained, and may have consisted mainly of iodinated conarachin.

Tests of the various antisera with the corresponding non-iodinated proteins showed that the heterologous reactions described above were dependent on the iodination of the protein and not on its normal reactivity. Thus, antisera to iodinated ox plasma reacted with normal ox serum, but not with casein or Ardein, antiserum to iodinated

Ardein with Ardein, but not with ox serum or casein, and antiserum to iodinated casein with casein but not with ox serum or Ardein. In other words, iodination had extended rather than altered the specificity. To this extent the results may be different from those of Clutton *et al.* [1938] who showed that the introduction of thyroxine or di-iodothyronine into the albumin or globulin molecules completely changed their specificity. It is possible, however, that the iodinated proteins contained a proportion of unchanged material so that the antiserum would naturally contain antibodies against non-iodinated protein, in which case there would be no need to assume that antibodies to iodinated protein would react with the parent substance. This explanation is in keeping with the result of another experiment in which an antiserum to iodinated Ardein, after being fully absorbed with Ardein, still reacted with iodinated Ardein.

In comparing the results with those of Harington and his co-workers, it must be remembered that in the iodinated proteins, as contrasted with the thyroxyl proteins, thyroxine constitutes only a small part of the iodine-containing constituents, since there is a very high acid-insoluble iodine content in relation to biological activity, and also a considerable content of acid-soluble iodine in organic combination. The new reactivity conferred by iodination is no doubt due in part to the presence of thyroxine acting as a haptene, especially as the vigour of the heterologous reactions is correlated with the biological activity of the iodinated proteins. In part, however, it is no doubt due also to the presence of other substances containing acid-insoluble iodine, but having little or no biological activity. Groups containing acid-soluble iodine in organic combination, e.g. di-iodotyrosine, may also presumably play some part.

*Antisera to iodinated proteins with thyroid preparations.* Under the conditions of the test, antiserum against each of the three types of iodinated proteins failed to give a definite precipitate with solutions of thyroid preparations, except that antiserum to iodinated plasma 91973 showed slight reaction with ox thyroid preparations. This antiserum naturally gave a strong reaction with normal ox serum, so that the slight reaction with the corresponding thyroid preparation is not of any great significance.

*Antisera to thyroid preparations with thyroid preparations.* These tests added nothing to existing knowledge; there was almost complete lack of species specificity. Good reactions were obtained with all combinations except those involving preparations of horse thyroids or antisera thereto; these seemed to be much less effective antigenically than those of other species. Antisera to thyroid preparations from all four species reacted strongly with homologous normal serum.

*Antisera to thyroid preparations with iodinated proteins.* Tests of these combinations were not exhaustive. The only definite positive reaction obtained was between antiserum to ox thyroid and iodinated ox plasma, a reaction which probably had no connexion with the iodination of the plasma. Even the best antigen among the iodinated proteins, iodinated Ardein DT/S/834, failed to react consistently with the antisera to thyroid preparations.

#### ASSAY OF PRECIPITATES

The work on precipitin reactions described above was directed mainly towards determining optimal proportions, so that precipitates could be made on a large scale and collected for assay. Owing to the amounts of serum required it has not been possible to do many large-scale precipitations, but enough results have been obtained to show that the precipitates are biologically active in the tadpole test. This means

that some, at least, of the active material is removed by precipitation, and that the tadpole is able to use some, at least, of the active material of the antigen-antibody complex, either because of the spontaneous dissociation of the latter in the tadpole medium or by its ingestion and digestion.

In a first experiment, 25 mg. of iodinated plasma N4 was precipitated with 75 ml. of homologous antiserum. The precipitate weighed 70 mg. and had appreciable thyroidal activity on tadpoles, but the amount available was inadequate for a proper assay. A more elaborate experiment was then carried out, the same iodinated protein being precipitated with varying amounts of another batch of homologous antiserum. Details are shown in Table 2. Increasing amounts of antiserum, up to 4 ml./mg. of

Table 2. *Assay of precipitates*

Test no.	Antigen		Antiserum		Precipitate	
	Nature	Amount (mg.)	Against	Amount (ml.)	Weight (mg.)	Biological activity
6	Iodinated plasma N4	25	Iodinated plasma N4	75	70	Slight
13	Iodinated plasma N4	40	Iodinated plasma N4	20	7	Slight
		40		40	22	Some
		40		80	43	Slight
		40		160	96	Some
152	Ox thyroid 2	15	Ox thyroid 2	60	20	Equivalent to about 3 mg. of ox thyroid 2
155	Iodinated Ardein DT/S/834	25	Iodinated casein CB1 + 2	25	21	Equivalent to about 4 mg. of DT/S/834
187	Iodinated Ardein DT/S/834	100	Iodinated casein CB1 + 2	10	30	0.19 (0.13-0.29)
		100		25	72	0.16 (0.11-0.23)
		100		50	87	0.15 (0.098-0.22)
		100		100	80	0.11 (0.075-0.165)
203	Iodinated Ardein DT/S/834	200	Iodinated casein CB1 + 2	10	Trace	—
		200		20	5	—
		200		50	135	0.17 (0.14-0.20)
		200		100	225	0.13 (0.11-0.16)
		100		100	160	0.07 (0.06-0.09)

antigen, gave increasing amounts of precipitate. All the precipitates showed some activity in the tadpole test, but the material was again inadequate for a proper assay. It seemed, however, that even with the large amount of antiserum used, the whole of the active material was not being recovered in the precipitate. A similar conclusion emerged from the next two experiments (Table 2, tests 152 and 155) in which ox thyroid preparation was precipitated with homologous antiserum, and iodinated Ardein with antiserum to iodinated casein. In both cases, only some 20% of the biological activity was detected in the precipitate. By this time, highly active serum from a sheep immunized with iodinated casein was available in some quantity. This permitted two larger scale experiments (tests 187, 203) in which enough precipitate was obtained from several combinations of antigen and antiserum to permit an adequate assay of the activity recovered. The results are shown in Table 2 in which the activity of the precipitates is shown, taking that of the parent antigen as 1.0, together with the limits of error for  $P = 0.95$ .

Increasing the ratio of antiserum considerably increased the total amount of precipitate, and, over the range in which assays could be made, there was a distinct tendency for the larger precipitate to be less active per mg. The total activity recovered, however, was sometimes greater in the larger precipitates. The activity detected in the precipitates was never more than a small proportion of that originally present. Three possible explanations may be offered of this result: (a) that destruction of active substances took place during the manipulations—this is unlikely; (b) that the tadpoles were unable to obtain all the active substance combined in the precipitates—this is not unlikely, if the activity of the precipitate depends on spontaneous dissociation of antigen and antibody in the tadpole medium; (c) that the active substance was only partially removed by the precipitation. For reasons explained in the next section, it was not possible to assay the supernatant fluids for residual active substance.

#### ATTEMPTS TO EFFECT PASSIVE IMMUNIZATION OF TADPOLES

Various attempts were made passively to immunize tadpoles against iodinated protein by pre-treating them with antisera. In all cases, treatment consisted in adding antiserum to the medium for 3 days before the usual test for thyroidal activity was carried out with known amounts of antigen. Normal rabbit serum was used for control experiments. The first experiments, carried out with antisera to iodinated Ardein and to iodinated plasma were quite unsuccessful. The addition of even 4 ml. of serum per 200 ml. medium led to very rapid bacterial growth in beakers maintained at 26° C., and as a result the tadpoles mostly died before the end of the 3 days. Changing the medium daily during the 3 days pre-treatment with serum allowed a few experiments to be concluded, but the technique was wasteful and time-consuming and no evidence was obtained of passive immunization. At a later stage in the research it was found that the addition of sodium sulphathiazole to the medium to make a concentration of 1/1000 was very effective in suppressing bacterial growth in the presence of serum, and attention was again directed to this problem of passive immunization.

Several experiments were carried out with the highly active sheep antiserum to iodinated casein. The tadpoles were put into a medium composed of 200 mg. of sodium sulphathiazole, 4 ml. of antiserum or normal serum, and 196 ml. of water. After 3 days the tadpoles were removed to clean water and tested in the usual way by giving graduated doses of iodinated casein for 3 days and reading the results 4 days later. The results, on experiments involving some 250 tadpoles, made it quite evident that no striking degree of passive immunization had been achieved. The last experiment suggested that pre-treatment with antiserum might have had some slight inhibitory effect on the subsequent response as compared with pre-treatment with normal serum, but the suggestion was so slight that the expenditure of a great quantity of serum and tadpoles would have been necessary to substantiate or refute it.

#### SIMULTANEOUS ADMINISTRATION OF ANTIGEN AND ANTIBODY TO TADPOLES

In some early experiments, iodinated plasma N4 of known activity was added to the water containing the tadpoles, together with serum from a rabbit chronically injected with the same preparation, in the hope that the presence of antibody, if any, would be shown by the formation of an antigen-antibody complex inactive on the tadpoles; in other words, that the effect of the iodinated plasma would be inhibited. This type of

experiment proved to be a complete failure. As in other experiments involving the use of serum, the water containing the tadpoles became very turbid and many of the tadpoles died during the 3 days period of treatment. This mortality was not much reduced by changing the medium and doses each day during treatment. Those that survived to the 7th day, at which the result of the test is normally recorded, showed at least some response to the iodinated plasma. Similar experiments using iodinated Ardein N4 SF and homologous antiserum were similarly unsuccessful. A repetition of these experiments, using antigen and normal rabbit serum led to similar results, except that the turbidity of the water and the consequent death rate were slightly reduced.

The subsequent use of sodium sulphathiazole enabled this difficulty of bacterial growth to be overcome, but other complications immediately appeared. It was found that the sulphathiazole inhibited to some extent the response of the tadpoles to iodinated casein, so that more than double the amount of active substance was required to produce the same effect on the tadpoles in the presence of 1/1000 sulphathiazole. A further difficulty was also encountered. For the demonstration of the effect under consideration it was necessary to show that administration of antiserum simultaneously with the iodinated casein inhibited the action of the latter and that normal serum had not this effect. Tests, however, showed that the addition of 4 ml. of normal serum to the medium containing sulphathiazole caused a further marked inhibition of the action of the iodinated casein, so that more than four times the amount of active substance was required, in the presence of serum and sulphathiazole, to produce the usual effect. In other words, both sodium sulphathiazole and normal serum gave an effect superficially similar to that sought in the antiserum.

No explanation of this curious result has yet been evolved, but presumably the presence of serum in the water inhibits in some way the absorption of active substance by the tadpoles. The results showed that it would be very difficult, by this method, to demonstrate special inhibitory activity on the part of the antiserum. They also showed that special precautions would be required in any attempt to use the tadpole technique for the assay of thyroïdal substances in circulating blood.

#### SUMMARY

1. Rabbits were injected daily for long periods with iodinated plasma, iodinated Ardein or iodinated casein. The sera of such animals reacted *in vitro* with the heterologous as well as with the homologous antigens, the vigour of the heterologous reaction being roughly proportional to the biological activity of the antigen. These sera did not, however, react with thyroid preparations.

2. Similar antisera were produced against preparations of the thyroid glands of various species. These reacted well with either the homologous or the heterologous thyroid antigens, but negligibly or not at all with the iodinated proteins.

3. Precipitates, collected on a fairly large scale from reactions between iodinated proteins and antisera thereto, showed thyroïdal activity in the test on *Xenopus* tadpoles, but only a small amount of the total original activity of the antigen was detected in the precipitates.

4. Attempts to effect passive immunization of the tadpoles by treatment with antiserum were unsuccessful, as were attempts to show an inhibitory effect of the antisera when administered to tadpoles simultaneously with the iodinated proteins.



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# ANTIGONADOTROPHIC ACTIVITY IN MALE RATS

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Hypophyseal or placental gonadotrophins may produce considerable ovarian growth and even giant ovaries [Hamburger, 1938; Uyldert & Freud, 1941], but if treatment is continued it eventually becomes ineffective, as a refractory state develops after periods which are characteristic for each gonadotrophin. These periods are roughly 14 days for chorionic gonadotrophin, 25 days for horse hypophyseal gonadotrophin, and 33 days for serum gonadotrophin (pregnant mares' serum). The ultimate ovarian size obtained is proportional to the dose and duration of treatment preceding development of the refractory state [Uyldert & Freud, 1941]; when the latter is once established all ovarian growth ceases or regression occurs in spite of continued treatment.

Not only do the gonadotrophins differ with respect to the periods necessary to produce refractoriness but also in the specificity of the refractoriness they produce [Parkes & Rowlands, 1936, 1937; Rowlands, 1937]. Refractory animals, such as rats or rabbits, yield sera which, injected into immature rats together with the antigenic gonadotrophin, will inhibit the latter's action on the ovaries. The ovaries resulting from such treatment are only slightly larger than those of untreated controls, contain no corpora lutea, and produce no oestrogen to enlarge the genital accessories or produce vaginal oestrus. This is an example of passive or transferable refractoriness. The antigenadotrophin appears to prevent the production of new follicles and corpora lutea rather than to cause those already present to regress.

The response in the gonads of males and females to gonadotrophic stimulation is very different, for while the ovaries may be increased in weight 20–25-fold the testes rarely show much gain in weight unless they have already regressed—as after hypophysectomy. Another striking difference is that the male rats do not apparently develop an overt refractory state during prolonged treatment with gonadotrophins, for their testes and accessory reproductive organs maintain sizes greater than those in normal adults and do not regress as the ovaries and accessory female organs do. The purpose of the experiments recorded here has been to investigate this peculiarity in some detail.

## MATERIAL AND METHODS

### *Hormones*

*Chorionic and serum gonadotrophin.* Commercial preparations of Pregnyl and Gestyl were kindly given us by N. V. Organon, Oss, Holland.

*Horse hypophyscal gonadotrophin* had to be produced in the laboratory. The gonadotrophin from this species is more efficient in male rats than that prepared from sheep or hog hypophyses. The efficiency of these last two in female rats can be enhanced by the addition of augmenting substances [Freud & Dingemans, 1941], but it was

preferred to avoid this complication. Such augmentation is not shown by the other gonadotrophins, but beef gonadotrophin is not sufficiently potent for use in anti-gonadotrophin investigations.

### *Activity of gonadotrophins*

The placental gonadotrophins were assayed by comparison with the International Standards and the horse hypophyseal gonadotrophin was used in doses producing similar responses. The activity of Pregnyl was 11  $\mu$ g. per i.u.; of Gestyl 30  $\mu$ g. per i.u., while 3.5 mg. of the hypophyseal extract represented 10 mg. of the original acetone-dried gland (10 mg.-equiv.).

### *Animals*

The rats used were all reared in the laboratory, fed a constant diet, and so far as possible divided so that litter-mates were present in experimental and control groups that were to be compared.

### EXPERIMENTAL

#### *Absence of overt refractoriness in males*

In Table 1 are recorded the results of experiments where male rats were injected with chorionic, hypophyseal, or serum gonadotrophin daily for 13-56 days. There was an acceleration of testicular growth and androgen production in the early phases of the

Table 1. *Continuance of enhanced androgen production during 56 daily injections of gonadotrophin in male rats*

Days treatment	No. of rats	Average body wt. (g.)	Wt. of testes (mg.)	Wt. of seminal vesicles (mg.)	Wt. of ventral prostate (mg.)
Controls					
(13-15)	1	69	500	19	27
(24-25)	2	103	1175	47	51
(31-33)	2	143	1275	92	51
(43-46)	3	179	2150	359	121
(55-56)	3	225	2433	642	310
Chorionic gonadotrophin (10-60 i.u. daily)					
13-15	4	70	700	213	112
24-25	4	97	1163	880	299
31-33	6	120	1817	632	214
43-46	4	182	1700	1425	558
55-56	4	222	1756	1913	911
Horse hypophyseal gonadotrophin (10 mg.-equiv. daily)					
13-15	3	76	940	378	82
24-25	3	110	1360	422	173
31-33	2	138	1760	416	191
43-46	4	181	2012	860	327
55-56	3	219	2025	1192	494
Serum gonadotrophin (60 i.u. daily)					
15	1	105	1300	600	269
20	1	135	1600	850	407
25	1	104	1850	1100	330
32	1	152	2350	1800	316
37	1	166	2800	1750	496
46	1	167	2700	1950	500
56	1	218	2800	2000	1000
66	1	180	2450	2050	500

experiments and no evidence that the accelerated androgen production had in any way diminished even after 56 days. There is some indication in the experiments with chorionic and horse hypophyseal gonadotrophins of some terminal regression from the maximum testicular weight achieved.

This is very different from the results of similar injections in female rats, where the refractory state is demonstrable not only in cessation of ovarian growth but also in the regression of uterine development and the production of dioestrus.

When allowance is made for the fact that the doses of these different gonadotrophins cannot be strictly equated the results agree very well. There is apparently no close relation between testis weight and the weight of the accessories though the prostate and seminal vesicle weights themselves are roughly proportional to each other [cf. Gaarenstroom & Freud, 1938]. Body weight was not affected by any of the three gonadotrophins.

The peculiar feature of these results is that in spite of the apparent continued action of the gonadotrophin injected, the sera of the male rats injected for longer than 33 days with either the chorionic or hypophyseal gonadotrophin contain anti-gonadotrophic activity if injected with the antigen into immature female rats (see below). There is thus an apparent difference in the developed antigonadotrophic activity in males and females in that the activity is demonstrable against endogenous gonadotrophins in the females but not in the males.

#### *Formation and maintenance of gonad tissue distinguished*

The maintenance of existing structure is apparently a gonadotrophic function separate from that involved in the formation of new tissue. If the refractory state involves this latter function only, then this would explain the sex difference, for apparently the formation of new tissue in the ovary is a more essential gonadotrophic function in the female which explains the greater demonstrable effects of gonadotrophin on the ovaries than on the testes. This would lead to the supposition that the endocrine function of the male depends on existing interstitial and intratubular elements of the testis while the secretory function of the ovary is dependent on the formation of new tissue.

Support for this view was obtained in an experiment where chorionic gonadotrophin injections were discontinued on the 56th day and the male rats were killed on the 66th day together with controls which had been injected for the whole period. Those whose treatment had been discontinued for 10 days had significantly lighter accessory reproductive organs, showing that even at this late stage androgen secretion is still being stimulated by exogenous gonadotrophin even though antigonadotrophin is demonstrable in the serum.

One objection to these interpretations may be that the injections do not directly stimulate androgen secretion but do so indirectly by stimulating the hypophysis: but if this were so then hypophysectomy after the 33rd day of treatment (when antigonadotrophin is first demonstrable) should produce genital atrophy in spite of the continued injections of exogenous gonadotrophin. An experiment was therefore carried out where some rats injected with 10 i.u. of chorionic gonadotrophin daily were hypophysectomized on the 33rd day. The results in Table 2 show clearly the atrophy following the operation where treatment was discontinued at the time of the

operation and the maintenance of the enlarged glands and organs in the animals where treatment was continued for the further 7-13 days until death. Since chorionic gonadotrophin is distinguishable from all other gonadotrophins (including serum gonadotrophin) in that it cannot produce testicular development in the absence of the rat's own hypophysis, the experiment demonstrates that the transferable anti-gonadotrophic activity does not interfere with testicular maintenance by exogenous or endogenous gonadotrophin.

Table 2. *Organ weights in rats injected with 10 i.u. of chorionic gonadotrophin daily and hypophysectomized after 31 or 36 days and killed 7-13 days later*

No. of rats	Duration of injections	Body weight (g.)			Wt. of testes (mg.)	Wt. of seminal vesicles (mg.)	Wt. of ventral prostate (mg.)
		Start	At hypo.	Death			
Control rats							
17	Till death	37	126	169	1728	723	259
3	Till hypo. of exp. rats	31	132	195	1817	533	179
Hypophysectomized rats							
18	Till death	37	132	130	1696	968	210
16	Till hypo.	36	143	138	966	171	45

### *Theoretical discussion*

It is interesting to speculate how the above considerations and results may be related to theories of the separable nature of gonadotrophic extracts. The current views may be summarized by saying that ovarian follicular, and testicular tubular, development depend on the follicle-stimulating factor while luteinization and the development of testicular or ovarian interstitial cells depend on the luteinizing factor. Now it has always been difficult to classify chorionic gonadotrophin within these terms since it stimulates testicular interstitial cells both morphologically and functionally in normal and hypophysectomized rats. This should class it as a luteinizing factor but such a view is contradicted by its failure to cause luteinization in the female after hypophysectomy. That it does maintain interstitial cells after hypophysectomy in the female might suggest its classification as an interstitial-cell-stimulating hormone were not the latter factor on other grounds now regarded as identical with the luteinizing hormone. The production of follicle stimulation and haemorrhagic follicles in the Aschheim-Zondek pregnancy test in mice has been shown in our laboratory [van Eck & Freud, 1938] and elsewhere to be doubtful or absent in the absence of the hypophysis. These difficulties do not affect the other gonadotrophins concerned since their action in hypophysectomized rats and mice is comparable with that in intact ones and follicle-stimulating and luteinizing factors are claimed to have been chemically isolated from—they though not by any universally agreed procedure.

Turning now to the differences in antigonadotrophic action recorded above, instead of considering the various end actions represented by follicle stimulation, luteinization and interstitial cell changes it may be more profitable to consider gonadotrophic action to consist of two functions of which these are merely the end results under varying circumstances.

We consider these functions under the headings of eutrophy and angiotaxis. *Eutrophy* is regarded as a visible improvement in the condition of specifically

responding gonad cells, excluding the gametes since oöcytes and sperm beyond the spermatocyte stage are not directly influenced by gonadotrophins (unpublished results). *Angiotaxis*, on the other hand, entails the formation of new vascularized responding tissue. Specific cells form new tissues where these can be adequately vascularized and these cells must therefore attract capillaries, hence the term *angiotaxis*.

Hypophyseal and placental hormones have antigenic properties which are slow to develop and therefore they have various reactions with the rapid angiotactic or eutrophic actions. Apparently the angiotactic aspect is interfered with more often or more demonstrably than the eutrophic one.

In these terms we may consider chorionic gonadotrophin as almost lacking in angiotactic action except in high doses [Pencharz, 1940; Simpson, Evans, Fraenkel-Conrat & Li, 1941]. Hence the failure to form new gonad tissues when this gonadotrophin is injected into hypophysectomized rats while the formation in intact rats depends on endogenous gonadotrophin. In the male, angiotactic action is slow compared with eutrophic action so that here the difference in intact rats between chorionic and other gonadotrophins is much less conspicuous than in the female and also refractoriness is not so apparent, as this condition chiefly involves the angiotactic function.

#### *Transferable refractoriness*

In spite of the failure to produce overt refractoriness in injected male rats all these animals developed antigonadotrophic activity in their sera. Table 3 shows the results of antigonadotrophic assays carried out on such sera.

Table 3. *Demonstration of transferable antigonadotrophic activity in the serum of male rats injected daily with chorionic gonadotrophin; method detailed in text*

		Antigonadotrophic assay				
Source of serum	No. of rats	Body wt.		Wt. of ovaries (mg.)	No. of corpora lutea per ovary	Oestrus
		Start (g.)	End (g.)			
Rats injected with 10 i.u. daily for 31 days*	4 Exp.	33	46	10	0.1	—
	6 Control	34	47	27	1.3	—
Rats injected with 30 i.u. daily for 66 days	4 Exp.	33	50	15	0.0	—
	4 Control	37	52	22	3.6	+
Normal (N) or hypo. (H) rats injected with 10 i.u. daily for 38 days†	3 Exp. (N)	25	47	9	0.0	—
	5 Exp. (H)	29	47	10	0.0	—
	5 Control	29	44	17	3.7	+
Normal (N) or hypo. (H) rats injected with 10 i.u. daily for 46 days	5 Exp. (N)	36	55	14	0.0	—
	5 Exp. (N)‡	33	48	13	0.0	—
	5 Exp. (H)	35	53	14	0.0	—
	5 Control	35	51	19	4.6	+
Experiment with horse hypophyseal gonadotrophin						
Rats injected with 10 mg. equiv. daily for 66 days	4 Exp.	34	54	21	3.2	+
	4 Control	39	57	84	99.2	+

\* Rats included in Table 1.

† Rats included in Table 2.

‡ Serum from rats injected for 36 days and untreated 10 days before death.

*Method.* Antigonadotrophic activity was tested by giving immature female rats 0.4 ml. of the serum daily for 4 days in eight injections together with total doses of

10 i.u. of chorionic gonadotrophin or 15 mg. equiv. of horse hypophyseal gonadotrophin. Control immature rats were injected with the same dose of the antigenic gonadotrophin alone. Daily vaginal smears were taken and the corpora lutea counted at death after crushing the ovaries between glass slides [Freud & Dingemanse, 1941]. The weights of the rats and of their gonads and accessory organs were also recorded.

*Results.* The increase in ovarian weight in the control groups (injected with antigen alone) is chiefly due to increases in the number of follicles or to the formation of corpora lutea, that is to angiotaxis. The luteinization was particularly marked with the hypophyseal gonadotrophin.

It is obvious that though the gonads of the donor rats were still stimulated even after 66 days of gonadotrophin treatment yet their sera contained antigonadotrophic activity as early as the 31st day of treatment. This activity is similar to that developed in female rats by similar treatment. The figures also show in confirmation of earlier authors [Selye, Bachman, Thomson & Collip, 1934; Selye, Collip & Thomson, 1934; Collip, Selye & Thomson, 1934] that the hypophysis is not essential for the development of antigonadotrophic activity and that this activity has not appreciably diminished even when the treatment has been discontinued for 10 days before withdrawing the serum. In experiments where inactive serum was given or too low doses were used the recipients developed oestrus, but where this occurred the serum was not necessarily ineffective, since ovarian growth and corpus luteum formation may still be inhibited. This emphasizes the conception that the transferable refractoriness interferes with the formation of the new tissue and angiotaxis rather than with the formation of hormones *per se*.

*Antigonadotrophic assay in male rats*

In young male rats androgen secretion is more closely associated with formation of new tissue than it is later and this led us to expect that the response of the accessory glands in such rats to treatment with chorionic gonadotrophin would be markedly

Table 4. *Demonstration of antigonadotrophic activity in the serum of male rats injected daily with chorionic gonadotrophin*

		Antigonadotrophic assay				
Source of serum	No. of rats	Body wt.		Weight of		
		Start	End	Testes	Seminal vesicles	Ventral prostate
		(g.)	(g.)			
4-day test						
Intact (N) or hypo. (H) rats injected daily with 10 i.u. for 36 or 49 days, all being killed on the 50th day	3 Exp. (N36)	35	49	388	11	24
	3 Exp. (N49)	39	54	429	12	28
	3 Exp. (H36)	37	54	443	26	47
	3 Exp. (H49)	37	53	431	10	25
	3 Control	37	51	460	32	56
6-day test						
Intact (N) or hypo. (H) rats injected daily with 10 i.u. for 32 or 40 days, all being killed on the 41st day	3 Exp. (N40)	37	49	250	9	19
	3 Exp. (H32)	35	47	217	9	18
	3 Exp. (H40)	40	53	292	11	20
	3 Control	40	53	417	28	63
	3 Control, un-injected	39	50	350	11	24

inhibited by the appropriate antiserum. An antigonadotrophic assay was therefore carried out in the same way as in the females (Table 4). The antigonadotrophic action is (with one exception) evident in the 4-day experiment when the weights of the prostate and seminal vesicles are considered but does not affect the gonad weight. When a 6-day experiment was carried out, with consequent 50% increases in total doses of antigen and antiserum, the expected effects on gonad weight were produced. The 4-day experiment appears also to show that transferable refractoriness diminishes faster in hypophysectomized rats than in intact ones, being almost lost during the fortnight that treatment was discontinued.

#### *Duration of treatment needed to develop transferable refractoriness*

In addition to those reported in Table 3, several other groups of rats of either sex were tested to ascertain the exact duration of the pre-treatment necessary to produce transferable refractoriness to the action of chorionic gonadotrophin. Treatment for 15 or 18 days was inadequate but transferable refractoriness developed between the 25th and 32nd days of treatment. This period does not significantly disagree with that required in female rats as we have earlier reported, so we may conclude that although overt refractoriness is not apparent in the male rats as it is in the female rats, yet their rates of development of transferable refractoriness are equal.

#### *Spontaneous refractoriness and the problem of senility*

The results so far described indicate that chorionic gonadotrophin elicits a refractoriness to an activity which it does not itself possess if we accept the view that it does not directly stimulate new tissue formation. The angiotactic activity which chorionic gonadotrophin induces in intact animals is due to endogenous gonadotrophin secretion and we therefore must assume that chronic injections of this gonadotrophin into intact rats produce a condition of refractoriness to rat gonadotrophin. This conception has been tested in the following experiments.

The results in Table 5 show the effects produced when antigonadotrophin produced by daily injections of chorionic or horse hypophyseal gonadotrophin into male or female rats for 30–32 days was tested against rat hypophyseal gonadotrophin. It may be seen that the action of the latter was not inhibited by the antisera but on the contrary was augmented. This demonstration of progonadotrophic action [Rowlands, 1938; Thompson, 1937] was unexpected. It should be emphasized that these sera, in spite of their progonadotrophic action with the rat gonadotrophin, showed full antigonadotrophic action against their antigens.

This production of a state modifying the actions of endogenous gonadotrophin led us to consider whether animals may become refractory to their endogenous gonadotrophin and whether such a condition might explain the facts of senility. The following experiment was designed to examine this theory.

About 90 immature male rats were injected daily for 4–8 days with a total dose of 4–8 ml. of serum\* from male or female rats more than 18 months old, or used as

\* An extract of serum was used which, in spite of the removal of much inactive material, would still only dissolve in the original volume of the serum. Active material was removed by 90% acetone precipitation, dissolved at pH 10, brought to pH 4.7 with sulphuric acid and left in the refrigerator overnight; the active material was precipitated by pouring the supernatant into four volumes of ethanol.



Table 5. *Assay of potent anti-(chorionic)-gonadotrophin and anti-(horse hypophyseal)-gonadotrophin against rat hypophyseal gonadotrophin (RHG)*

Total dose of antiserum (ml.)	Total dose of RHG (mg.-equiv.)	No. of rats	Body wt.		Wt. of ovaries (mg.)	No. of corpora lutea per ovary	Wt. of uterus (mg.)	Oestrus
			Start (g.)	End (g.)				
Anti-(chorionic)-gonadotrophic serum								
1.6	15	4	26	42	45	15.5	59	+
0.0	15	5	26	37	33	12.1	57	+
1.6	0	2	27	44	11	0.0	24	—
1.6	15	4	42	60	49	27.0	56	+
0.0	15	4	41	61	18	1.4	56	+
1.6	0	4	40	62	14	0.0	31	—
Anti-(horse hypophyseal)-gonadotrophic serum								
1.6	10	4	37	61	63	26.6	74	+
0.0	10	4	36	62	30	12.5	64	+
1.6	15	3	49	69	82	37.3	76	+
0.0	15	4	43	66	62	30.8	86	+
1.6	0	2	51	75	12	0.0	27	—
1.6	15	3	43	61	29	9.3	58	+
0.0	15	3	43	57	15	0.0	66	+
1.6	0	3	41	55	9	0.0	19	—

control rats and not injected at all. The results are given in Table 6. Where the difference in the means was more than 3 times the square root of the sums of the squares of the probable errors the difference was regarded as significant. Eight other rats were injected with pooled serum from immature rats but the number was too small for statistical evaluation though the results showed that the serum had no effect on general health or genital development.

Table 6. *Effect of injections of serum from senile rats into immature male rats*

Group	Body wt. at death (g. $\pm$ p.e.)	Wt. of testes (mg. $\pm$ p.e.)	Wt. of seminal vesicles (mg. $\pm$ p.e.)	Wt. of ventral prostate (mg. $\pm$ p.e.)	Wt. of adrenals (mg. $\pm$ p.e.)
Experimental	53.2 $\pm$ 2.1	309 $\pm$ 27	8.2 $\pm$ 0.7	19.0 $\pm$ 1.2	26.2 $\pm$ 1.5
Uninjected controls	56.3 $\pm$ 2.2	439 $\pm$ 27	9.7 $\pm$ 0.8	22.6 $\pm$ 1.0	23.9 $\pm$ 1.0
Difference in means	3.1	130	1.5	3.6	2.3
$3\sqrt{(p.e._1^2 + p.e._2^2)}$	9.1	115	3.3	4.8	5.4

The results show a significant difference in testicular weight indicating some inhibition of tissue formation by the serum from the old rats. Thus the experiment lends some support to the suggestion that senile conditions may be at least in part due to the development of antigonadotrophic activity against endogenous pituitary secretions.

## SUMMARY

Male rats show no loss of gonad or accessory reproductive organ weight when injected daily for 56 days with chorionic, serum, or horse hypophyseal gonadotrophin though their sera antagonize the actions of these gonadotrophins in immature rats. The development of this transferable refractoriness is similar in males and females.

When gonadotrophic treatment of normal or hypophysectomized male rats is interrupted the genital accessory organs regress, showing that the injected gonado-

trophin is still acting although their serum contains antigonadotrophic activity demonstrable in immature female recipients.

This antigonadotrophic action can be demonstrated in male recipients providing immature rats are used.

It is suggested that gonadotrophic action is classifiable into two types: (a) the maintenance of existing tissue and its function—eutrophic action, and (b) a stimulation of the production of new gonadal tissue—angiotactic action. Chorionic gonadotrophin has no angiotactic action; antigonadotrophic action affects angiotactic action but not eutrophic action.

Transferable antigonadotrophic activity develops after 25 days' treatment with chorionic gonadotrophin.

Chronic treatment with chorionic gonadotrophin or with horse hypophyseal gonadotrophin leads to the development of serum augmenting the action of rat hypophyseal gonadotrophin.

Young male rats injected with serum from old rats showed an inhibited testicular growth suggesting that senility may be connected with the development of antigonadotrophic activity to endogenous pituitary secretion.

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# FACTORS INVOLVED IN MAINTAINING INVOLUTION OF THE THYMUS DURING SUCKLING

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That the involuted thymus gland of pregnancy regenerates after delivery was first pointed out by Fulci [1913a] and confirmed by Bompiani [1914], Ohmura [1928], and Jolly & Lieure [1930]. Bompiani also reported that if delivery is followed by lactation the involution is maintained throughout the suckling period (though not in the guinea-pig [Jolly & Lieure, 1930], presumably owing to the irregular and poor nursing in this species). When the litter is removed regeneration of the gland sets in and is rapidly completed.

Bompiani [1914] gave no information of the age, number, nor of the variations in body weight or thymus weight of the animals he used and the first object of the present work has been to confirm and extend Bompiani's results by collecting quantitative data on the rates of involution after parturition and weaning (21 days after delivery). The second object has been to investigate the possible influences of the sex hormones on the involution by comparing it in weaned and suckling females after spaying.

During the foregoing experiment incidental findings that the size of the litter, number of functional nipples, and milk retention apparently influenced the rate of involution led to an investigation of these factors, and of the changes in other endocrine organs dependent on them.

## METHODS

*The animals* used were 104 albino female rats of our inbred strain all of which were primiparous and nearly uniform in weight and size. All but eleven of them were about 4 months old when mated and where possible litter-mates were evenly distributed among the different experimental groups.

A liberal *diet* was available to all animals, since insufficient food causes a rapid involution of the thymus, while Brody, Riggs, Kaufman & Herring [1938] have reported the striking increase in food intake occurring with the onset of lactation.

*The body weight* of the animals was recorded daily and the averages are shown in Fig. 1.

*Organ weights* of the thymus, thyroid, adrenal, and pituitary glands, of the spleen and of two iliac lymph-nodes were recorded after death by torsion balance after fixation in Bouin-Hollande-sublimate. The twelve mammary glands were dissected (except in weaned females) and weighed before fixation.

*Histological studies* of samples of all these tissues were made and measurements of the various components of the thymus (cortex, medulla, and connective tissue) and of the spleen were obtained by Hammar's [1926] and Hellman's [1930] methods of quantitative analysis.

## RESULTS

The average data for the whole series are recorded in Table 1. As there is a fairly close relation between thymus weight and body weight the relative weight of the organ has been chiefly used (expressed as g./kg. body weight) so that differences in the average body weight of the different groups do not affect the results. In the

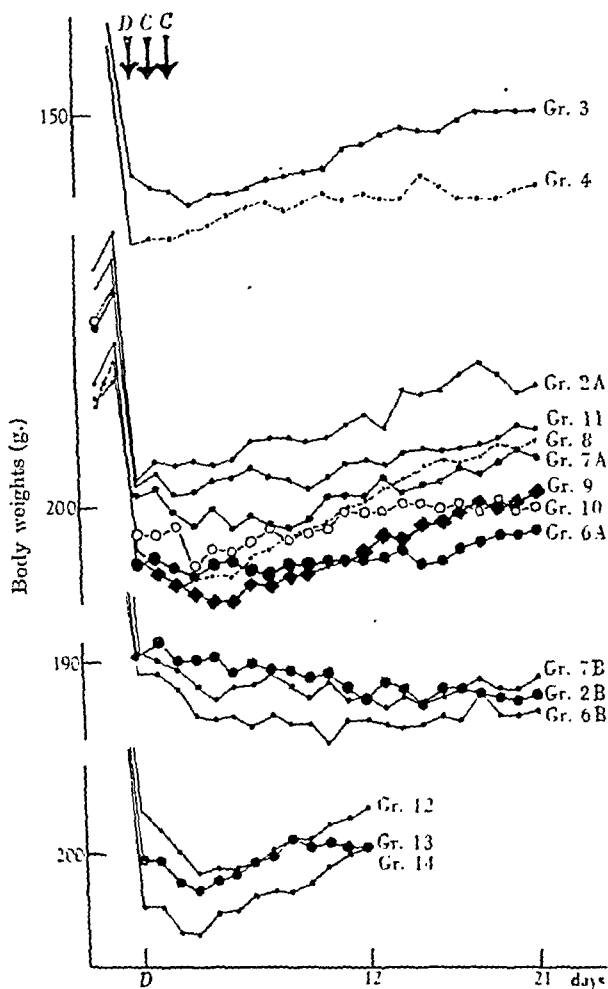


FIG. 1. Curves of the average daily body weights (in grammes) from 2 days before delivery until the 21st (above) and the 12th (below) days of lactation. Arrows: D, delivery; C, castration (Groups 1-14).

graphs the relative weights of the cortical tissue, which is the most labile component of the thymus gland, have generally been used. Since any loss in body weight rapidly leads to reduction in thymus size, those animals that gained weight (subgroups A) are separated from those that lost weight (subgroups B). The statistical significances of any differences reported have been established by calculation of the probable error of the difference of the means. Probable errors are included in the table in border-line cases only.

*Maintenance of thymus involution during suckling*

When the weights of the thymus gland were compared in females killed at delivery of litters of 10 young (Group 1) and in females which had suckled 10 young for 21 days and were then killed (Group 2A) the only significant difference was that the thymus medulla was lighter in the latter group. These results confirm Bompiani's contention that the involution is maintained during suckling. It may also be noted that the weights were in all cases smaller in the suckling rats that lost weight (Group 2B) than in those that gained weight (Group 2A).

*Maintenance of thymus involution during suckling in spayed rats*

In a preliminary experiment 8-month-old females were spayed on the 1st or 2nd day after parturition: the rats in Group 3 had their young removed at the same time while those in Group 4 suckled litters of 10 young each. The total weights of the thymus and of all parts other than the interstitial tissue were four to five times greater in the former group when all were killed 21 days after delivery (Fig. 2). The gain in body weight in the two groups during the 21 days was the same. Exactly similar results are shown when Groups 6A and 9 are compared.

When intact and spayed rats suckling equal litters are compared (Groups 2 and 6) the thymus weights among those gaining weight (the A subgroups) are slightly lower in the spayed rats (Group 6A), but when the total groups are compared then the weights are slightly lower in the intact rats. Neither difference is statistically significant.

These results demonstrate that the regeneration of the thymus occurring when suckling is stopped and the maintenance of the involution during suckling both take place in spayed rats and are therefore independent of ovarian secretions.

*Relation between thymus regeneration and size of litter*

Among the rats used in the preliminary experiment of the preceding section one was exceptional in killing all her young but one. Data for this rat (Group 5) showed that the weights of the thymus were about three times greater than in the rats suckling 10 young. An experiment was made to confirm this chance observation.

Thirty-three 4-month-old female rats were spayed on the 2nd or 3rd day after delivery and divided into four groups, suckling 10, 5, 1 or 0 young per mother. All were killed 21 days after parturition and the four groups all showed fairly steady increases in body weight in this period (Fig. 1) though comparison of the A subgroups shows that the mothers suckling 0 or 1 young gained more weight than those suckling 5 or 10.

The results in the table (Groups 6-9) and in Figs. 3-6 show that the average weights of the thymus gland (parenchyma, cortical and medullary tissue) were greater in the mothers with less young. These differences cannot be attributed to differences in body growth since the same relations were seen in the B subgroups, where all the



FIG. 2. Size of the thymus (reduction to  $\frac{2}{3}$ ), 21 days after parturition. Above: spayed and weaned females (Group 3). Below: spayed females having suckled 10 young (Group 4).

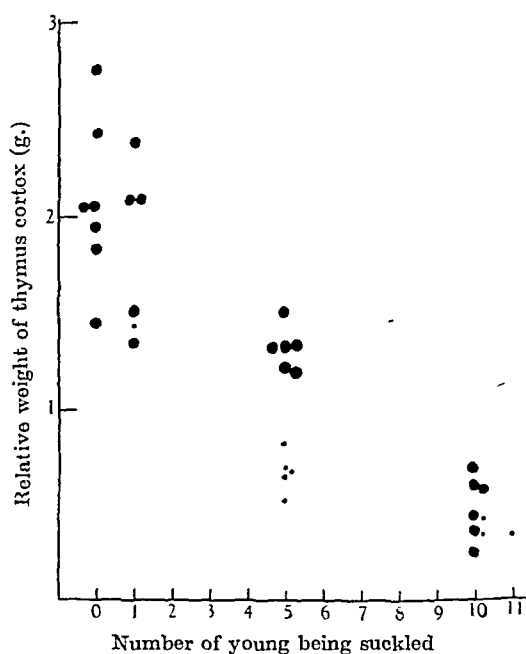


FIG. 3.

FIGS. 3-8. Relations between relative weights of thymus cortex (Figs. 3, 5, 7) and thymus medulla (Figs. 4, 6, 8), plotted in ordinates, and number of young having been suckled (Groups 6-9), weight of the litter (Groups 6-11) and number of functional nipples (Groups 6, 9, 10, 11) plotted in abscissae. (The data recorded by females having lost in body weight are given by small circles in all figures.)

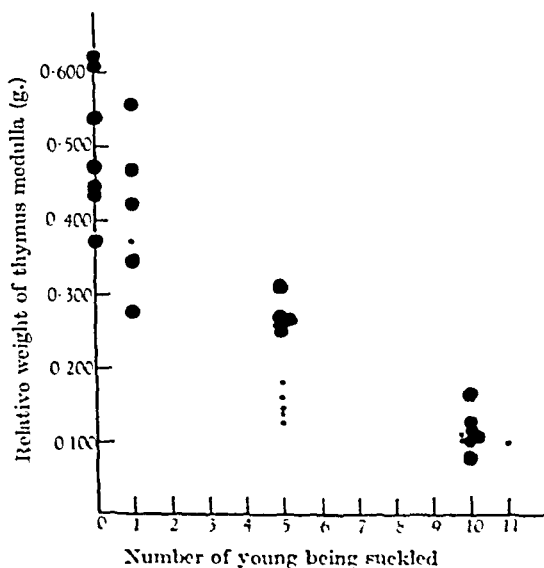


FIG. 4.

females showed slight loss in body weight and the thymus weights were accordingly lower than those in the corresponding A subgroups.

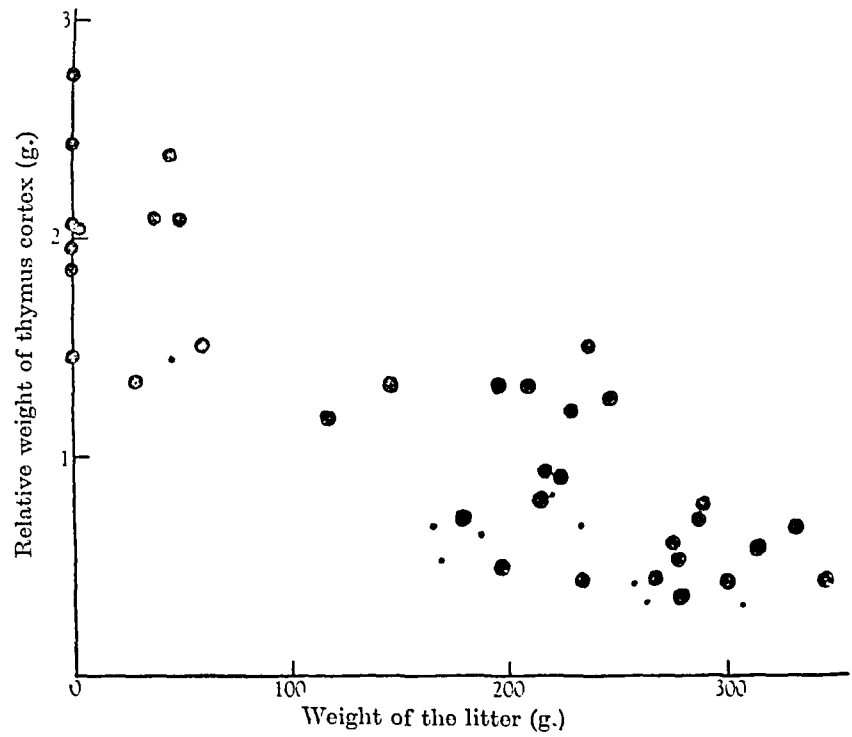


FIG. 5.

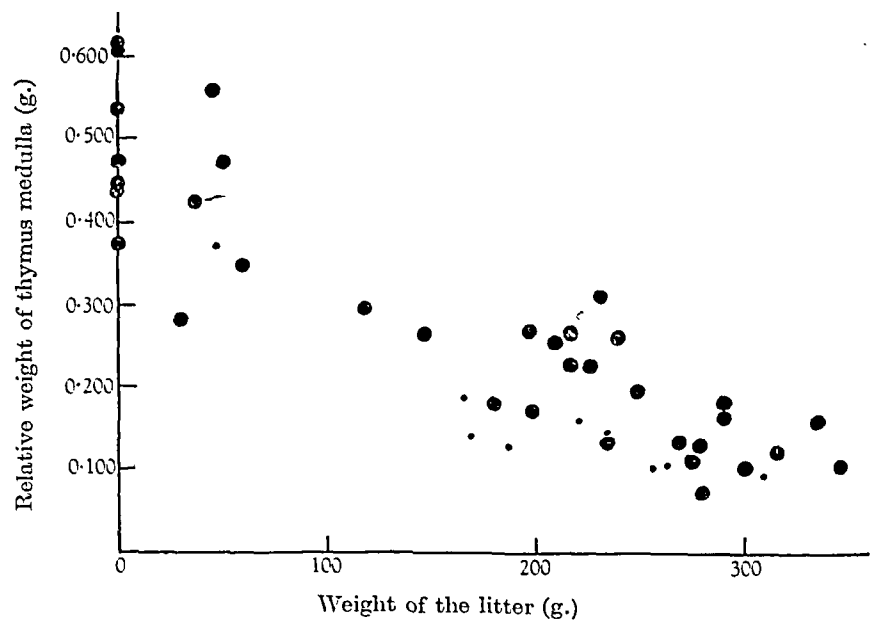


FIG. 6.

These figures leave little doubt that there is a relation between thymus regeneration and the size of the litter being suckled, though associated with the different size of the litter are corresponding differences in litter and mammary gland weights.

*Relation between thymus regeneration and number of functional nipples*

One of the mothers suckling 10 young in the preceding experiment (in Group 6A) had an exceptionally heavy thymus gland when killed; about double that of the

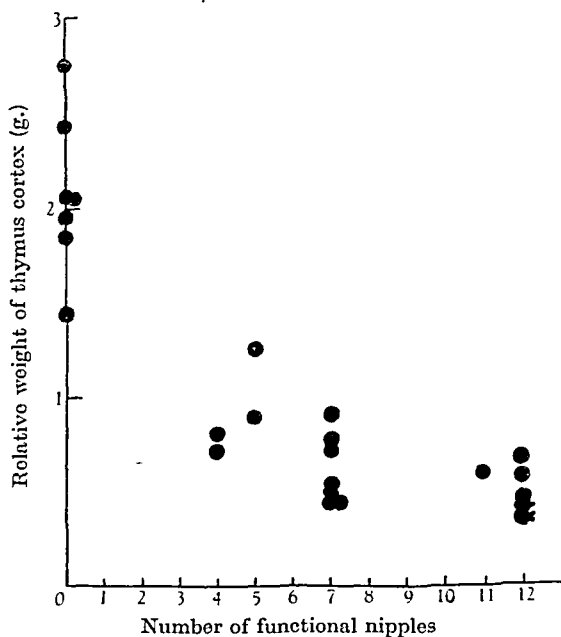


FIG. 7.

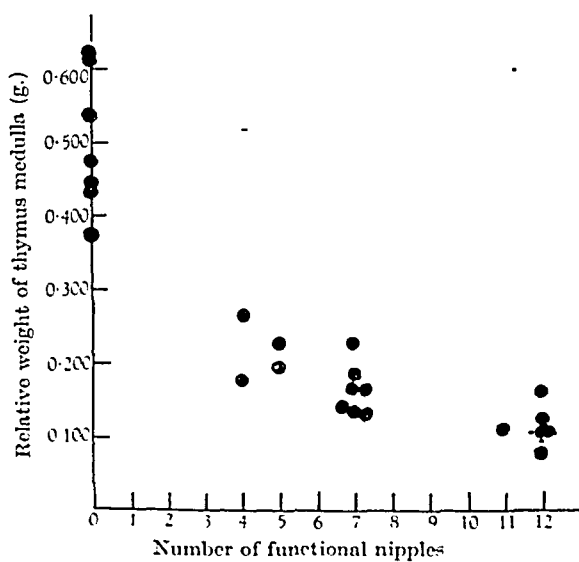


FIG. 8.

other members of the group. This rat was peculiar in having only five hypertrophied nipples; seven were absent. This suggests that the rate of thymus regeneration may be related to the number of nipples used or to the strength of the suckling stimulus.



It should be possible, by destroying some nipples without impairing the growth of the litter or the total amount of milk drained, to establish whether the withdrawal of milk or the mechanical stimulation of the nipples is responsible for maintaining thymus involution. Such a procedure, according to Selye, Collip & Thomson [1934], shows that suckling of the remaining nipples prevents the unused mammary glands from involution.

In the experiment recorded in Groups 6A, 10 and 11 females were allowed to suckle 10 young through 12, 5, or 7 nipples. All the rats were spayed on the 2nd or 3rd day after parturition and killed on the 21st day. The weights of the thymus gland were heaviest in the mothers with the lowest number of nipples (Group 10) and the two factors seemed to be related (Figs. 7 and 8). The differences in the gains in body weights of the three groups were not significant nor was there any difference in the weights of the mammary tissue between Groups 6A and 10 though that in Group 11 was significantly lower. Unfortunately, however, this experiment is not completely satisfactory as the litters of the mothers with only 5 or 7 nipples did not grow so rapidly as those in the mothers with all 12 nipples. The weights of the litters were 32 and 21 % less in Groups 10 and 11 than in Group 6A, while the weights of the thymus were 77 and 23 % greater.

#### *Effect of strong nipple stimulation without milk drainage*

The foregoing results may be equally explained by differences in the strength of the suckling stimulus (due to different litter sizes or to different numbers of functional nipples) or by differences in the amount of milk withdrawn. Further information on this point was obtained by using the observation of Selye [1934], Selye *et al.* [1934] and Selye & McKeown [1934] that continued suckling maintains the secretory activity of the mammae even when removal of the milk is prevented by cutting the galactophores.

Three groups of female rats were spayed 2-3 days after parturition. Those in Group 12 had their young removed soon after delivery, those in Group 13 suckled large litters, while those in Group 14 had the main galactophore cut in each nipple at, or a few days before, delivery.

This operation was carried out by making a skin incision along the costal line on the inner side of the nipple so that the cutaneous nerves were avoided and the main milk duct was then drawn out with a small hook and cut. If the duct is not thus fully exposed it easily escapes the scissors. The completeness of the section was confirmed histologically and by inspection of the stomach in the young rats.

The rats in Groups 13 and 14 were paired so that there were the same number of young in each. The young ones were exchanged each day between the mothers of the two groups so that they had access to milk on alternate days.

The mothers were all killed on the 12th day after delivery at which time mammary involution had not taken place [Selye *et al.* 1934]. All the rats in Group 14 were vigorously suckled throughout the experimental period. When killed, their nipples were enlarged and elongated and the area beneath the nipple had become a thick spherical bulge of folded skin. The weight of the mammary tissue in the rats with cut galactophores was 56 % of that in the normally suckling rats.

The females in Group 14 that had lost no milk gained in body weight more than those in Group 13 but the difference was small compared with the difference in thymus weight and so is not significant (cf. Fig. 19). The relative and absolute weights of the thymus gland in the three groups were significantly different, being lowest in the suckling rats of Group 13 and highest in the weaned rats (Group 12); the relative proportions between Groups 13, 14 and 12 were roughly 1 : 2.5 : 4 when the cortical weights were compared. Group 15 comprised three rats originally intended for Group 14 but in which some of the operated nipples became necrotic and disappeared so that the suckling stimulus in these rats was only applied to 3, 6 and 1 nipples (nos. 1450, 1453, and 1455).

Evidently milk withdrawal retards thymus regeneration but by itself does not prevent this occurring, for suckling stimulation alone has also some retarding effect.

### *Histological examinations*

#### *Thymus glands*

The relative proportions of cortex, medulla and interstitial tissue in the gland under different conditions were mostly not significantly altered, nor were there any striking morphological changes. Even in the most involuted glands the normal appearance was maintained: the cortex was crowded with small lymphocytes (though the cells were sometimes not so densely packed as normally) and the border between cortex and medulla was still clearly defined (Fig. 9); the layer of connective tissue separating the lobules remained thin.

Epithelial tubular and cyst-like structures connected with medullary and interstitial tissue were seen in some glands, but as they were irregularly present in various experimental groups no functional significance could be attached to them. Hyperaemia was marked in the cortex and connective tissue of the glands in the group where milk drainage was prevented.



FIG. 9. Section of thymus of female 1454 (Group 13) having been suckling 15 young for 21 days. Maintenance of normal histological picture in spite of a striking reduction in size and weight (0.080 g.).

#### *Lymphoid tissue*

The iliac lymph-nodes were studied since these are not directly under the influence of mammary conditions. The weight of these organs was higher in weaned females or in those suckling small litters than in those suckling large litters, but the differences were only questionably significant. Hypertrophy of the regional lymph-nodes during pregnancy has already been reported [Hellman, 1930], and the weights recorded here in the pregnant rats (Group 1) were significantly higher than in lactating females (Group 2). The changes in the weight of the spleen are somewhat greater than in the lymph-nodes and the relative weight of the lymphoid tissue was significantly greater in the weaned females. Red and white pulp appeared to be equally affected. On the whole, in spite of individual variations, the germinal centre appeared bigger in both spleen and lymph-nodes of the weaned females and there appears to be some parallelism

between the thymus reactions and those of the lymph tissue generally although the latter is on a much smaller scale.

### *Mammary glands*

Detailed descriptions of the changes in the mammary glands will be reported elsewhere. Here it is sufficient to describe the appearance of those glands whose nipples were destroyed, not used, or undrained. In these cases the mamma appears as a flat yellowish cake crossed with milk-filled ducts, the main one being swollen until almost spherical. The gland substance was somewhat involuted but clearly distinguishable from the glands of the weaned animals in which only a few remnants of collapsed alveoli and ducts remained scattered in adipose tissue.

### *Changes in thyroid glands*

The weight of the thyroid gland is not directly proportional to body weight in rats [Brody & Kibler, 1941; Mixner, Bergman & Turner, 1943], but as no figures are available for the relation of thyroid to body weight in the rats used here and as females of different body weight are distributed evenly among the experimental groups, the direct relative weights are given in the Table. Another difficulty in assessing the effects of different treatments on thyroid function is the large histological variation between individuals of the same group, between the two glands of the same animal, and in the relative sizes of the active central part and inactive peripheral part of the same gland. There may even be a direct discrepancy between two criteria of activity—heightened epithelium may be present at the same time and place as a high storage of colloid. These qualifications must be remembered in considering the results of the 12,000 measurements of epithelial height recorded in Figs. 10 and 11 (epithelial height was measured in 150–200 follicles in each of 90 rats).

### *Thyroid histology*

Frequency distribution curves of the thyroid epithelial height in Groups 1 and 2 were almost identical, showing that suckling itself had little effect on thyroid histology in intact rats. When spayed suckling rats were compared with intact ones (Groups 6 and 2) the spayed rats showed more intense colloid absorption and greater epithelial height.

The frequency distribution curves in Fig. 10 fall into two groups. The group on the right, having the higher epithelia, consists of the spayed rats killed after 21 days of suckling 10 or 5 young through 12 nipples, or 10 young through 7 nipples (Groups 6, 7 and 11). That on the left, with the less active glands, comprises spayed rats killed after 21 days of suckling 1 or 0 young through 12 nipples or 9–10 young through only 5 nipples (Groups 8, 9 and 10); thyroid glands in these three groups appear completely inactive or almost so.

While there is little doubt that the difference between the two sets of curves is significant, the differences within each trio are probably not. It may be noted that there is some indication of a relation between number of young suckled and thyroid activity in that the curve of the rats suckling 5 young is to the left (except at the peak) of that of those suckling 10 young. Also, although the average curves of Groups 6 and 11 show no difference, only two of the glands in the group with 7 instead of 12 active nipples were hyperactive while 5 were inactive or only poorly active.

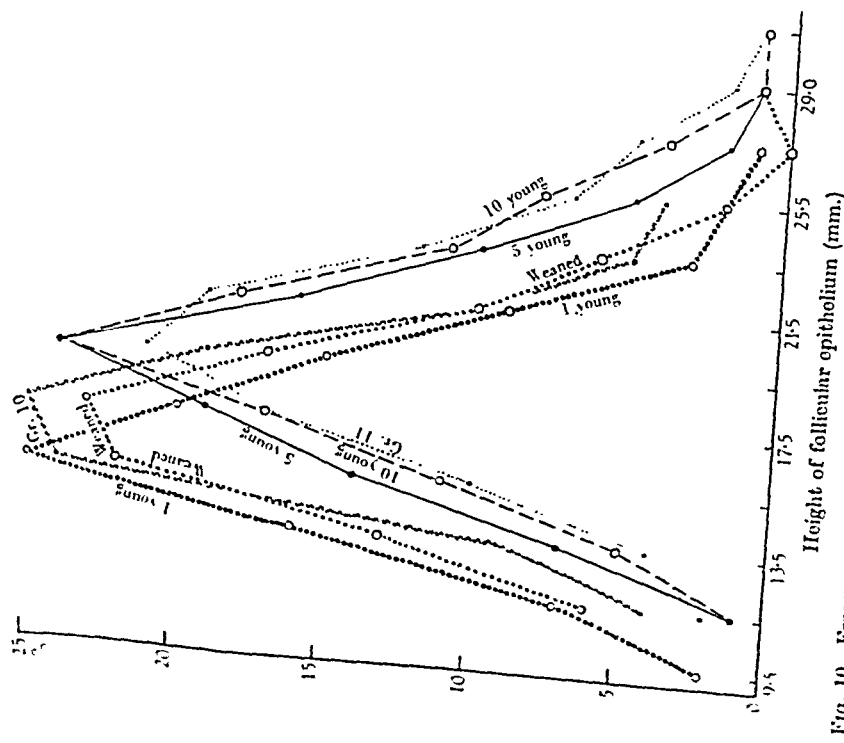


Fig. 10. Frequency curves of average percentage of the various heights of follicular epithelium in thyroid glands of Groups 6-11, plotted from 9300 measurements from camera lucida drawings (10 mm.  $\pm$  5  $\mu$ ). Explanation in the text.

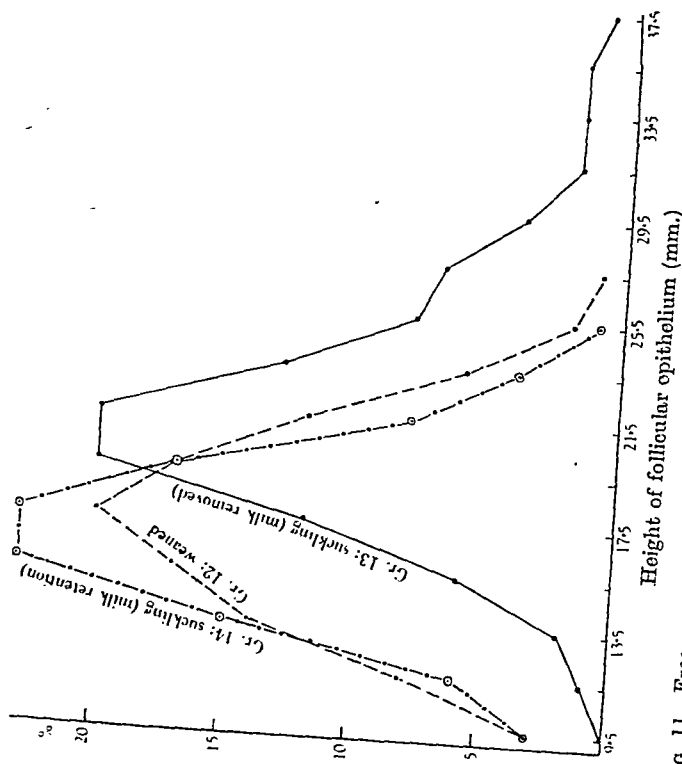


Fig. 11. Frequency curves (cf. Fig. 10) in thyroid glands of Groups 12, 13 and 14, plotted from 3300 measurements from camera lucida drawings. Percentage of low heights of follicular epithelium in thyroid glands of weaned females and of females actively suckling with milk retention strikingly higher than in those of females normally nursing.

Where the number of young suckled by a spayed female is unusually large, as in Group 13, thyroid hyperactivity is pronounced (see Fig. 11 and Pl. 1, fig. 13) after 12 days' suckling. The inactivity of the glands in spayed rats killed at the same time but having cut galactophores (Groups 14 and 15) or having had their young removed at birth (Group 12) is shown in Fig. 11 and illustrated in Pl. 1, figs. 12 and 14 (most of the glands in Groups 14 and 15 were even more inactive than that illustrated).

### *Thyroid weight*

In intact rats the thyroid was significantly heavier after 21 days' lactation than it was at delivery.

In spayed rats 21 days after delivery the gland was heavier after suckling than when litters were removed at birth. This is clearly shown by comparing Groups 6, 7, 8 and 9. (The fact that the difference, though present, is not significant between Groups 3 and 4 may be due to the greater age of the rats or because they were examined in winter; their histological differences were also less marked.) There was no close relation between thyroid weight and size of litter or number of functional nipples.

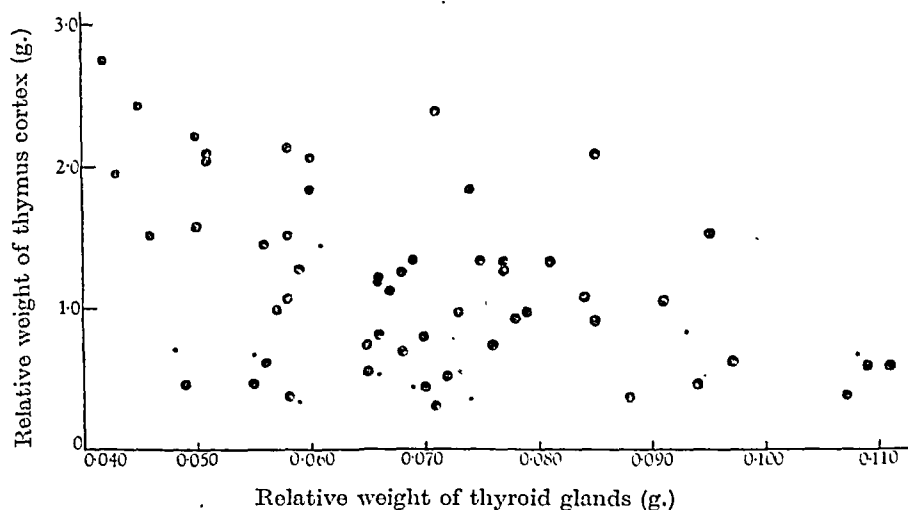


FIG. 15. Relation between relative weights of thymus cortex (in ordinates) and of thyroid glands (in abscissae) (Groups 6-15).

Where the spayed rats were killed 12 days after delivery (Groups 12-15) the same differences were noted. In those rats whose galactophores were cut the thyroid weight was slightly and questionably higher than in the weaned group but strikingly lower than in the normally nursing group.

In Fig. 15 the relative weight of the thyroid gland in all the spayed groups is plotted against the relative weight of the thymus cortex. There is no correlation between the two.

### *Changes in the adrenal weight*

As changes in adrenal weight closely follow those in body weight [Mixner *et al.* 1943] the relative weight of the glands is referred to in the following statements.

In intact females the adrenals are significantly lighter after 21 days of suckling than at delivery (Groups 1 and 2A).

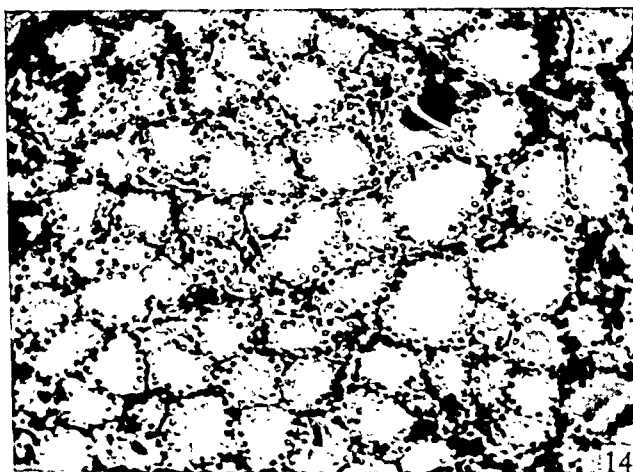
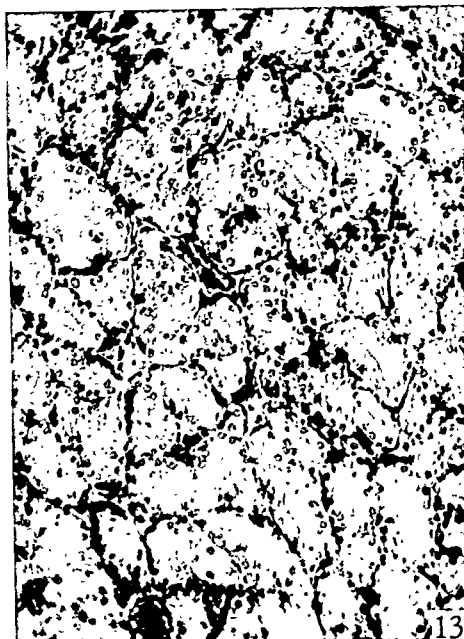
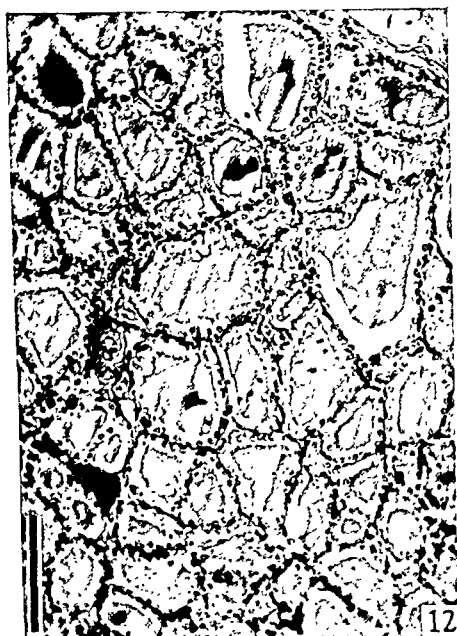


FIG. 12. Thyroid gland of female 1416 (Group 12) 12 days after delivery, spaying and weaning. Picture of functional inactivity: epithelium low, colloid storage, scarce vacuoles. Microphoto Panphot Leitz. Magnif.: the scale represents 100  $\mu$ .

FIG. 13. Thyroid gland of spayed female 1443 (Group 13), having been suckling 21 young for 12 days. Picture of strong activity: epithelium columnar, disappearance of colloid in the follicles. Magnif. as in Fig. 12.

FIG. 14. Thyroid gland of spayed female 1447 (Group 14, paired with 1443 of Group 13), with trans-  
section of each galactophore, and having been suckling 21 young for 12 days. Functional inactivity. Magnif. as in Fig. 12.



In spayed females suckling 10 young for 21 days the adrenals are heavier than in intact rats under the same conditions and than in spayed rats whose young were removed at birth. Adrenal weight is lower in spayed rats suckling 1 or 5 young for 21 days than in those suckling 10 young: the relation is graphed in Fig. 16. The number of functional nipples does not appear to bear any relation to adrenal weight.

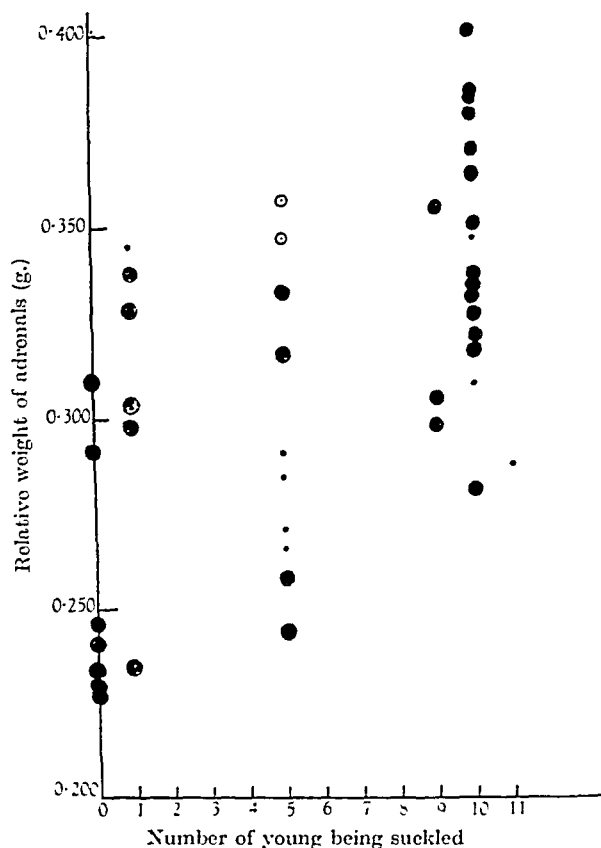


FIG. 16. Relation between relative weights of adrenals (in ordinates) and number of young being suckled (in abscissae) (Groups 6-11).

In the spayed rats killed 12 days after delivery the rats suckling abnormally large litters had adrenals 25 % heavier than in rats whose litters were removed at birth. The rats with cut galactophores had adrenals of intermediate weight.

In the graphs relating adrenal weight to the weights of thymus cortex or medulla (Figs. 17 and 18) in all the groups of spayed rats (Groups 6-15) there appears to be some degree of inverse proportionality in each case.

#### DISCUSSION

##### *The form of involution*

The histology of the thymus and the relative weights of its component parts show that the involution during pregnancy and lactation is a shrinkage of the gland without any alteration in its morphology. This absence of any striking histological change does not agree with the findings of Fulci [1913b] in the pregnant rabbit, where a loss



of the distinct separation of cortex and medulla, scarcity of lymphoid cells and the presence of 'infiltration' and 'degeneration' cells filled with lipid material were described. These changes closely resemble those found in accidental involution (loss of distinct cortico-medullary border, disappearance of cortical lymphoid cells, and phagocytic activity of the epithelial reticular cells) and as Fulci gives no data on the variations in weight of his material it is possible that accidental involution did in fact interfere with the changes due to pregnancy.

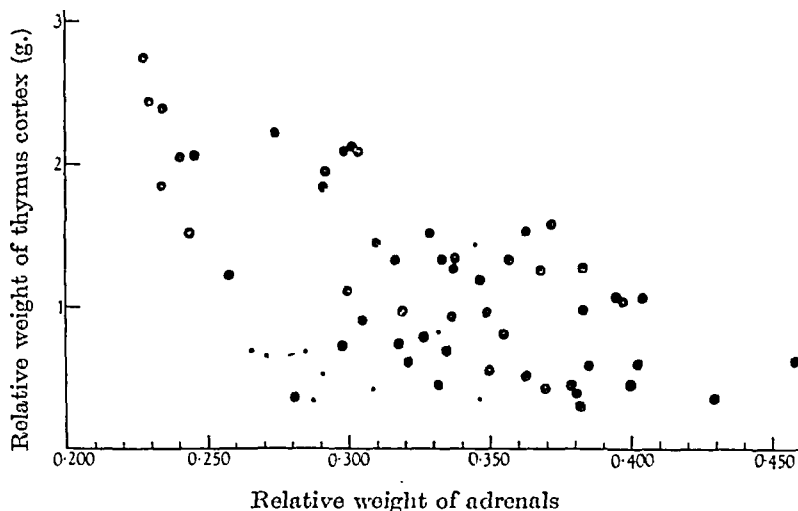


FIG. 17.

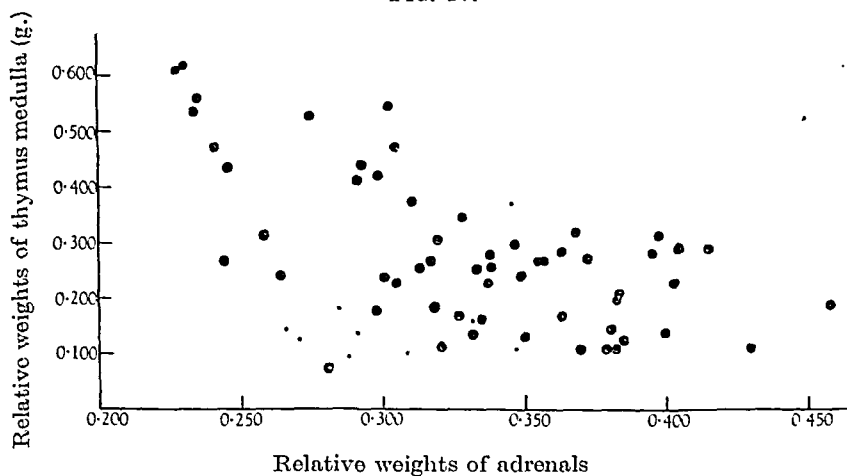


FIG. 18.

FIGS. 17, 18. Relation between relative weights of thymus cortex and medulla (in ordinates) and relative weights of adrenals (in abscissae) (Groups 6-15).

The pregnancy involution is also clearly distinguishable from that produced in ageing, which is characterized by the development of adipose tissue between the atrophic lobules of the gland. The only form of involution resembling that of pregnancy is that produced by moderate doses of thymus-depressing hormones which suggests that it is due to some form of hormonal influence.

There is some degree of correlation in this material between the behaviour of the thymus and that of lymphoid tissue generally. Such parallelism has been reported

under various experimental conditions. Verdozzi (1924) reported hyperplasia of the spleen pulp in lactating females but this we have been unable to confirm.

*Possible causes of maintenance of involution*

*Metabolic drain of suckling*

The partial, or completely normal, regeneration obtained when litter size or weight was reduced, when all the nipples were not functional so that litter growth was impaired, or when the galactophores were cut and partial mammary involution resulted might all be attributed to diminished expenditure of energy. Thus interpreted, the maintenance of thymus involution would be considered as a physiological 'accidental involution' similar in its consequences to that taking place in starvation, nutritional disturbances, infection, or cachectic disease. We have already seen that pregnancy involution differs histologically from accidental involution and the following objections may also be raised against this interpretation.

It is well known [cf. Zuntz, 1936; Young, 1938; Richter & Barelare, 1938; Barelare & Richter, 1938; Poo, Lew & Addis, 1939] that lactation involves a heavy strain on the mother and increases her needs of fat, carbohydrate, protein, and minerals (particularly of calcium and phosphorus). According to Brody *et al.* [1938] the total metabolism of the lactating rat may be twice that of the pregnant rat. However, adequate food supply will protect lactating females from any metabolic imbalance except for a loss of calcium. (We are unable to say whether a specific nutritional lack, such as that of calcium, would play any role in the thymus involution we are studying, though in thymectomy experiments there is no recent confirmation of earlier reports connecting the thymus with calcium balance.) Furthermore, there is evidence (see below) that increases in metabolism or in nutritional needs only produce thymic involution when the animals have a negative metabolic balance shown by loss of body weight. In our experiments the females have been in excellent nutritional condition as shown by their gains in weight (Fig. 1) and the slight variations recorded in most groups chiefly depend on daily fluctuations without physiological significance. When variations in body weight are compared with thymus weight within each group (Fig. 19) there is seen to be no close correlation except possibly in Group 7. We conclude that the increased metabolism and removal of milk regarded as generalized nutritional factors cannot be held responsible for the maintenance of thymic involution.

There remains the possibility that the involution may be maintained by the lack or deficiency of some specific material in spite of a generalized positive balance producing a gain in total body weight. Andersen, Prest & Victor [1937] have shown that the metabolism of different organs may be altered in different directions in the same lactating animal but they did not study the thymus. Dustin [1913] and Jolly [1923] suggested that the thymus functions as a store for nucleoprotein but Grégoire & Grégoire [1934] have shown that this reserve material is a negligible proportion of the total nucleoprotein in the body: the daily endogenous catabolism of nucleic phosphorus in the rabbit is two to four times greater than the total thymic reserve. Whether any other specific material is required for thymic regeneration we cannot say.

From a survey of the literature the only procedures able to produce atrophy of the thymus unassociated with loss in body weight appear to be the injection of adequate

amounts of thymus-depressing hormones. These are oestrogen [Chiodi, 1938], testosterone propionate [Chiodi, 1938; Freeman & Small, 1941; Grégoire, 1945], gonadotropin [Evans & Simpson, 1934], and adrenocorticotropin [Evans, Moon, Simpson & Lyons, 1938].

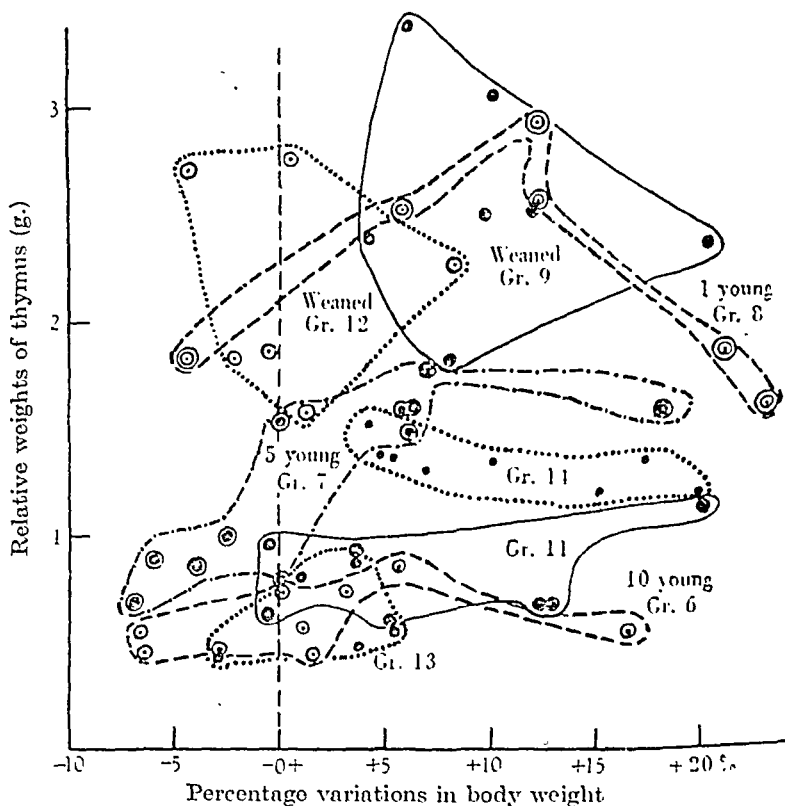


FIG. 19. Relation between relative weights of thymus (in ordinates) and percentage of increase (+) or loss (−) in body weight after delivery (in abscissae). The figures recorded in each group are surrounded by continued or interrupted lines.

### *Nervous factor of suckling*

Selye [1934], Selye *et al.* [1934] and Selye & MacKeown [1934] claim that stimulation of the nipples is an important factor in maintaining lactation, possibly by influencing prolactin production in the anterior pituitary. Such nipple stimulation could also account for the relation we have found between thymus regeneration and the number of suckled young or the number of functional nipples. The possibility was investigated in two experiments but the results were not entirely clear-cut and we conclude that both milk withdrawal and the nervous stimuli of suckling are factors concerned in maintaining thymic involution.

In the first experiment (Groups 6A, 10 and 11) the number of functioning nipples was reduced in some mothers. Unfortunately, the litters of these mothers, although of the same size as in the controls, did not gain weight so rapidly. The gains in weight between the 3rd and 21st day were 390, 320 and 240 % where the mothers had 12, 7 and 5 nipples. The 18 % impairment of growth in the litters of the mothers with 7 nipples was almost inversely proportional to the 23 % gain in thymus weight in these mothers, suggesting that milk withdrawal is related to thymus regeneration. The

mothers with only 5 nipples, on the other hand, showed a 77 % increase in thymus weight with only 38 % reduction of litter growth suggesting that suckling stimuli do play some part in thymic regulation.

The second experiment (Groups 12-14) concerned animals with large litters and cut galactophores. This experiment was partly unsatisfactory in that suckling from nipples whose galactophores were cut did not completely maintain secretory activity; involution of the mammary glands, although delayed as compared with that after weaning, was already apparent 12 days after delivery. This partial involution was also seen in the previous experiment in the mammae whose nipples were excised while suckling was maintained in the remaining nipples. This does not agree with the original findings of Selye [1934] and Selye *et al.* [1934], a failure also reported by Weichert [1942]. Twelve days after delivery in suckling mothers, the thymus cortex weighed 26 %, while in the mothers who were suckled without milk drainage it weighed 63 %, of that in weaned controls. If suckling stimuli play no part in thymus regeneration the two last weights should be equal. Inversely, if withdrawal of milk plays no part, the two first weights should be identical. In the event 63% is exactly midway between 26% and 100%.

The mechanism by which the milk drainage and the suckling stimulus can influence the thymus can at present only be conjectured but it is probable that the hypophysis is involved. It may therefore be worth while recalling certain similarities in the behaviour of the thymus and hypophysis during lactation. Thus Haterius [1932] showed that the histological changes in the hypophysis of pregnant females are maintained during lactation but the gland rapidly recovers its normal appearance after weaning. Desclin [1936] showed that the ovaries were not concerned in this, for lactating spayed females still had hypophyses typical of pregnancy with no castration cells while spayed females without litters had castration cells. As the most striking changes occurring during lactation affect the eosinophil cells, Desclin [1945] assumed that these cells were responsible for the production of prolactin. Desclin & Grégoire [1945] have shown that suckling without milk drainage is just as effective as normal nursing in maintaining the condition of the hypophysis as it is in pregnancy.

Another interesting parallel is between the rate of thymus regeneration and the lactogen content of the pituitary. Reece & Turner [1936-7] found the latter was three times greater in non-suckled rats than in suckled rats; the hypophyses of mothers who were allowed to nurse without milk withdrawal had an intermediate prolactin content. This result may be compared with the thymus weights in our Groups 12-14.

There is thus some basis for the assumption that prolactin secretion may be concerned in thymic regulation, either directly or through the intervention of other anterior pituitary hormones. The effects of prolactin injections on thymus and lymphoid tissue have not been extensively reported in the literature. Kemp & Marx [1937] observed 'eine beträchtliche relative Thymushyperplasie' in one dwarf mouse injected with prolactin during a short time, but the hormone used was of doubtful purity: Goldzieher, Sherman & Sherman [1942] injected different doses of prolactin and described decreases in spleen weight with enlargement of the perifollicular zone and increases in number and size of the reticular cells of the pulp; and Dougherty & White [1943] have reported slight decreases in the weight of the inguinal lymph-nodes in mice after prolactin but no significant weight changes in other tissues. We

intend to make a more thorough study to determine whether prolactin is capable of producing the thymic changes we have been investigating here.

*Possible influence of other endocrine factors*

*The ovaries.* The observations in spayed animals clearly show that ovarian secretions are not needed for the maintenance of thymic involution and the possibility that prolactin is an active factor by virtue of its luteotrophic properties [Astwood, 1941; Evans, Simpson & Lyons, 1941; Evans, Simpson, Lyons & Turpeinen, 1941; Lyons, Simpson & Evans, 1941, 1942, 1943] can also be ruled out.

*Thyroid glands.* Our findings in the spayed animals suggest that lactation is associated with thyroid hyperfunction. This agrees with Verdozzi's report [1924] of hyperfunction and hyperplasia of the thyroids of lactating guinea-pigs (though his histological descriptions of the gland hardly agree with his conclusions) and the marked hyperplasia reported in nursing bitches by Bernard [1927] and Mascia [1939]. Our findings also suggest that the degree of hyperfunction is related to the number of nursed young and functional nipples; active suckling without milk removal inhibits the glands.

The associated thyroid conditions cannot, however, be regarded as the cause of the maintained thymus involution since hyperthyroidism itself causes no such involution. Administration of thyroxine [Utterström, 1910; Courier, 1928; Grégoire, 1942*a*] or thyrotropin [Grégoire, 1940*a, b*, 1941, 1942*a, c*] with maintenance of a positive metabolic balance, as shown by maintenance or increase of body weight, produced no thymic involution even in young chicks showing a 3 to 6-fold increase in thyroid weight. The thyroid changes are presumably the expression of alterations in thyrotropin secretion [cf. Turner, 1939; Turner & Cupps, 1939]. In active suckling without milk drainage, inhibition of the glands is possibly due to diminished thyrotropin secretion due to milk retention.

*Adrenal glands.* Our finding that adrenal weights are slightly heavier at delivery than after 21 days' nursing agrees with that of Donaldson [1924] and not with those of Verdozzi [1924], Andersen & Kennedy [1932-3], Sleeth & Van Liere [1939] and others who report an additional hypertrophy of the cortex during lactation.

In our spayed rats there was a decrease in adrenal weight when the number of young was reduced or milk-loss was prevented by cutting the galactophores. These decreases are possibly due to diminished adrenocorticotropin secretion.

There is already ample evidence of a relation between the adrenal cortex and thymus and lymphoid tissue generally. The thymus and lymph nodes are enlarged after adrenalectomy (see literature reviewed by Grégoire [1943]) and there is involution of these organs in conditions of adrenal hypertrophy [Jackson, 1915, 1919; MacCarrison, 1921; Andersen, 1935; Selye, 1936; Dougherty & White, 1945] or following injections of adrenocortical hormones [Ingle, 1938, 1940; Ingle, Higgins & Kendall, 1938; Wells & Kendall, 1940] or of adrenocorticotropin [Evans *et al.* 1938; Dougherty & White, 1943; Simpson, Li, Reinhardt & Evans, 1943].

In the present experiments the maintenance of thymic involution during the suckling period and the variations recorded in its regeneration under different conditions are probably partly dependent on changes in adrenal function. The relations between thymus and adrenal weights graphed in Figs. 17 and 18 support this view.

of females in each group given in brackets

Iliac lymph-nodes		Spleen		Thyroid glands		Adrenals	
Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
0.0555	0.275	0.548	2.71	0.0117	0.058	0.0646	0.319
—	—	—	$\pm 0.33$	—	$\pm 0.0054$	—	$\pm 0.0135$
0.0317	0.135	0.691	2.91	0.0186	0.080	0.0639	0.278
—	—	—	$\pm 0.27$	—	$\pm 0.0044$	—	$\pm 0.0092$
0.0199	0.110	0.568	3.13	0.0129	0.071	0.0591	0.327
—	—	—	—	—	—	—	—
0.0251	0.121	0.623	3.03	0.0154	0.075	0.0612	0.305
—	—	—	—	—	—	—	$\pm 0.0094$
0.0204	0.135	0.580	3.76	0.0117	0.078	0.038	0.256
—	—	—	—	—	$\pm 0.0025$	—	—
0.0112	0.083	0.390	2.79	0.0110	0.082	0.041	0.309
—	—	—	—	—	$\pm 0.0033$	—	—
0.0075	0.046	0.503	3.07	0.0120	0.073	0.042	0.256
—	—	—	—	—	—	—	—



*Miscellaneous*

It may be pertinent here to recall that variations in number of young suckled by lactating rats are associated with other physiological variations such as those in oestrogen sensitivity [Parkes & Bellerby, 1926-7], in the length of gestation in inseminated lactating rats [Weichert, 1940] and in the size of the corpora lutea on the 21st day of lactation [Weichert, 1940].

We may also point out that the insignificant differences between females having one and no young are paralleled by Parkes's observation [1935] that dioestrus is not present during lactation when the litter consists of only 1-2 young.

## SUMMARY

1. The degree of involution of the thymus is the same in rats at delivery and after 21 days' suckling of a normal litter of ten.

2. The maintenance of this involution during suckling is unaffected by spaying soon after parturition.

3. The rate of regeneration of the thymus after delivery decreases with increases in the number and weight of the litter, weight of mammary tissue, and number of functional nipples.

4. Section of the galactophores in actively suckled females results in regeneration of the thymus during the lactation period but not to the same extent as in weaned females.

5. The histology of the involution at parturition and during lactation differs from that in other types of involution: ageing or accidental.

6. The changes reported are not associated with loss of body weight, growth upsets, or nutritional imbalance.

7. The reactions of the lymphoid tissue of iliac lymph nodes and spleen are similar but less obvious.

8. The histology of the thyroid glands of normal females at delivery and on the 21st day of lactation is the same. The thyroids of spayed lactating females were more active. The thyroids of spayed females suckling large litters were hyperactive; those of spayed females with litters removed at birth were inactive. The hyperfunction decreases with decreases in litter size and number of functional nipples. Prevention of milk escape in suckling spayed females inhibits the hyperfunction.

9. The adrenal glands are heavier at parturition than after 21 days' suckling, and in spayed lactating females than in intact lactating, or spayed weaned, females. In suckling females adrenal weight decreases with reduction in the number of young suckled. Prevention of milk withdrawal in suckling females leads to a decrease in adrenal weight.

## CONCLUSIONS

The general conclusion to be drawn from the discussion of the possible factors concerned in maintaining the involution of the thymus during lactation is that probably endocrine, and not metabolic, factors are primarily concerned. It appears likely that variations in secretion of anterior pituitary hormones (lactogen, adrenocorticotropin) produced by changes in suckling stimuli or in milk removal affect thymus regeneration either directly or by related changes in the secretion of thymus-depressing hormones.



An over-secretion of adrenocorticotropin appears to be a probable factor in maintaining the involution. There is no evidence that the thyroid glands are concerned in any way.

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# EFFECTS OF PROLONGED OESTROGEN ADMINISTRATION IN FEMALE NEW WORLD MONKEYS, WITH OBSERVATIONS ON A PERICARDIAL NEOPLASM

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Whereas monkeys of the Old World, especially rhesus, have repeatedly been the object of experimental research on the tumorigenic action of oestrogens the authors of the present work have no knowledge of papers dealing with experimental or spontaneous neoplasms in New World monkeys. Zuckerman [1940] mentions unpublished experiments of Russel & Zuckerman [1939] on a marmoset in which cystic glandular hyperplasia of the endometrium was produced.

Insular or nodular epithelial proliferation and metaplasia and likewise hyperplasia of muscular and conjunctive tissues have been induced by a prolonged treatment with oestrogens in the genital region of both sexes in macacus and baboon [for literature see Zuckerman, 1940; Engle, Krakower & Haagensen, 1943]. There are also statements about spontaneous tumours in different Old World primates [Engle & Stout, 1940].

The object of our research with New World monkeys was related to former findings in the guinea-pig subjected to a prolonged administration of oestrogens. Uterine [Nelson, 1937] and other abdominal fibroids were easily induced in the female guinea-pig [Iglesias, 1938; Lipschutz & Iglesias, 1938; Lipschutz, Iglesias & Vargas, 1940; Lipschutz, 1942]. On the other hand there are many statements which favour the concept that uterine fibromyomata in women may be due to a disturbance of the normal balance between follicular and luteal hormonal action and of their normal timing [Lipschutz, 1939]. This concept is based on the fact that uterine fibromyomata in women are often accompanied by thickening of the myometrium, by cystic glandular hyperplasia of the endometrium, uterine bleeding, and a cystic condition of the ovary [see also Hamblen, 1945, p. 529].

For the above reasons, work was undertaken on primates under experimental conditions similar to those that induce uterine or other abdominal fibroids in almost all the female guinea-pigs to which oestrogens were administered for 3 months. Simultaneously Vargas [1943] on behalf of this Department and independently Engle *et al.* [1943] have studied the same question in rhesus.

## MATERIAL AND METHODS

### *Animals*

Capuchin monkeys were available from Ecuador. They were classified according to Brehm's 'Tierleben' as *Cebus apella* [Brehm, 1922], but very probably some of the animals were crosses of different local varieties of Ecuador. They survive for years in apparent good health in the outdoor animal houses of the Department and also in the local Zoo.

Non-castrated females have been used throughout. Though the ovary has been shown in the guinea-pig to diminish the degree of the fibromatous reaction to oestrogens [Lipschutz, Murillo, Vargas & Koref, 1939] we have preferred to work with non-castrated primates in order to avoid interference by peritoneal scars. It was also thought advisable not to create experimental conditions too dissimilar from those in women with fibromyomata, which is a pre-climacteric disease.

#### *Administration of oestrogens*

Administration of oestrogens was adapted, as to the timing and quantities given, from our experience with the fibromatogenic action of oestrogens in the guinea-pig.

In the course of our work with guinea-pigs we have found that proper timing is fundamental if tumours are to be elicited: administration must allow for a *continuous* action of the oestrogen. This can be attained in two ways: by subcutaneous implantation of tablets [Deanesly & Parkes, 1938] or by injection of esterified oestrogens that are slowly absorbed [Miescher, Scholz & Tschopp, 1938]. With subcutaneously implanted tablets the quantities of  $\alpha$ -oestradiol necessary to induce abdominal fibroids in the guinea-pig are 50 to 100 times smaller than those which must be given when free  $\alpha$ -oestradiol is injected thrice weekly [Lipschutz, Thibaut & Vargas, 1942; Riesco, 1942, 1944]. Likewise the fibromatogenic activity of certain esters of  $\alpha$ -oestradiol, as for instance the 17-caprylate, is on injection about 50–100 times greater than the activity of the free hormone [Lipschutz, Bellolio, Chaume & Vargas, 1941]. Similarly, certain esters of artificial oestrogens revealed a very high tumorigenic activity when injected in oily solutions (the propionic ester of diethylstilboestrol [Bruzzone, 1942], the propionic ester of benzoestrol [Blanchard, Stuart & Tallman, 1943; Blanchard & Stebbins, 1945; Alvear, 1944]).

As seen in Table 1 the oestrogen was administered in the present work by subcutaneous implantation of tablets of  $\alpha$ -oestradiol and oestradiol dipropionate, pure or mixed with cholesterol (nos. 4 and 6). In three animals, injections of the highly fibromatogenic esters also were given twice weekly for 4–9 months before necropsy. The quantities injected per week were 200–400  $\mu$ g. of the 17-caprylic and of the 17-benzoic-3-*n*-butyric ester of oestradiol (nos. 5 and 3) and 400  $\mu$ g. of the propionic ester of benzoestrol (no. 6). In the guinea-pig abdominal fibroids can be elicited when about 20 mg. of the mentioned esters of  $\alpha$ -oestradiol are given weekly during 3 months [Lipschutz *et al.* 1941]. Even when taking into consideration the greater weight of the monkeys, the quantities injected were several times greater than those sufficient to elicit abdominal fibroids in the guinea-pig (for details see Discussion below).

Tablets were implanted beneath the skin of the back, at a certain distance from the scapula. The total absorption from the tablets was calculated from the loss of weight; for the mixed tablets calculation was based on the assumption of non-selective absorption [Shimkin & White, 1941; Fuenzalida, 1944]. Injections were made twice weekly into the thigh.

#### EXPERIMENTAL OBSERVATIONS

##### *The behaviour of the genital tract and of the mammary gland*

A summary of the microscopic findings is given in Table 2. There was stratification and cornification of the *vaginal mucosa* including the outer cover of the intravaginal uterine parts in all the animals treated. In several cases the stratified vaginal

Table 1. *Five female cebus monkeys treated with oestrogens*

No.	Weight		Total duration of experiment (days)	Details of administration of oestrogens				Total absorbed (mg.)	Absorbed per day ( $\mu$ g.)
	At start (g.)	At end (g.)		Days*	Oestrogen	Mode of administration	Weight of tablet, or quantity injected		
1	900	1400	203	<203 61	Oestradiol diprop. Oestradiol diprop.	Subc. tab. Subc. tab.	90.1 mg. 97.0 mg.	—† 41.5	—† 680
4	1020	770	254	254 98	$\alpha$ -Oestradiol Oestradiol diprop.	Subc. tab. Subc. tab.	108.8 mg. 352.4 mg.‡	48.0 19.4§	189 198§
3	2095	1780	291	291 66 63	$\alpha$ -Oestradiol Oestradiol benz.-but. Oestradiol benz.-but.	Subc. tab. Inject. Inject.	109.8 mg. 100 $\mu$ g., 2 x week 200 $\mu$ g., 2 x week	58.3 1.9 3.6	200 29 57
5	1900	2160	397	397 66 174	$\alpha$ -Oestradiol Oestradiol capryl. Oestradiol capryl.	Subc. tab. Inject. Inject.	101.8 mg. 100 $\mu$ g., 2 x week 200 $\mu$ g., 2 x week	69.8 1.9 9.4	178 29 57
6	2200	1580	1016	1016 860 261	$\alpha$ -Oestradiol Oestradiol diprop. Benzocetrol prop.	Subc. tab. Subc. tab. Inject.	101.9 mg. 370.9 mg.‡ 200 $\mu$ g., 2 x week	—† 26.0§ 15.0	—† 30§ 57

\* Figures in heavy type indicate how long *continuous*, or *uninterrupted*, oestrogenic action was *certain*.

† Tablet totally absorbed or more probably eliminated, at unknown date.

‡ Tablet consisting of mixture of 40 % oestrogen and 60 % cholesterol.

§ Reduced to pure oestrogen. Note that average absorption per day appears smaller in animal 6, which was implanted longer, than in animal 4. This is probably due to formation of a thick capsule around the hormone-cholesterol tablet.

Table 2. *Microscopic condition of genital tract in five cebus monkeys treated with oestrogens*

No.	Endometrium	Uterine glands	Cervical mucosa	Outer cover of intravaginal uterine parts	Vaginal mucosa	Observations
1	High cylindrical. <i>Insular metaplasia</i> . <i>Pseudostratification</i>	Cystic hyperplasia	Insular squamous metaplasia	Cornified	Cornified. Desquamated piece of endometrium in the lumen	Small cyst beneath the capsule of the spleen
4	High cylindrical. <i>Pseudostratification</i> . De-squamated endometrium in the lumen	Cystic hyperplasia. Great development of <i>polyps</i>	Insular squamous metaplasia surpassing height of fornices	Cornified. <i>Polyps</i>	Cornified	—
3	High cylindrical	Small cysts	Insular squamous metaplasia surpassing height of fornices	Cornified. Digitations	Cornified	Sclerotic excrescences of the splenic capsule
5	High cylindrical	Small cysts. <i>Polyps</i>	Insular squamous metaplasia	Cornified. Digitations	Cornified	—
6	High cylindrical	Highest degree of cystic hyperplasia. <i>Largo polyps</i> . Blood in the lumen of cysts	Squamous epithelium only at the entrance; higher up no metaplasia	Cornified. <i>Enormous digitations</i>	Not examined	Small fibrous induration in the diaphragm. <i>Adenocarcinoma of the pericardium</i>

mucosa was freed from the cornified layers which were still adherent to the mucosa of the intravaginal uterine parts.

The cornified digitations around the *uterine opening* offered a striking picture. They varied in the degree of development but they were present in all animals with the exception of no. 1, i.e. the animal with the shortest duration of oestrogenic treatment. In no. 6 the uterine opening offered a spectacular aspect even to the naked eye (Pl. 1, figs. 1 and 2). The whole part was covered with excrescences resembling superficially a cauliflower cervical tumour in woman (Pl. 1, figs. 2 and 3). Even in no. 4, in which no additional injections were given, the digitations were highly developed and were of a polypous aspect (Pl. 3, fig. 10).

The wall of the vagina was greatly thickened; this was due to the increase of the muscular layers and conjunctive tissue.

There are two important points to be held in mind when discussing the above changes: they were not metaplastic and they were not insular or nodular. Stratification and cornification of these structures is a normal event. But the growth in our animals was certainly a disorderly one as evidenced by figs. 2, 3 and 10 (Pls. 1 and 3).

On the other hand, metaplastic changes in the *cervical mucosa*, as known in rhesus since the work of Overholser & Allen [1933], Engle & Smith [1935], Hisaw & Landrum [1936], and Zuckerman [1937*a*], were present also in our monkeys. The picture in cebus seems to be like that in rhesus. The metaplastic changes were in our animals always insular (Pl. 2, fig. 6) as in the work of Engle & Smith [1935] and others on rhesus, i.e. the metaplastic nodules of the cervical epithelium were surrounded by hyperplastic glandular epithelium. The extension of these metaplastic changes of the cervical mucosa was very variable according to the animal. At the entrance the squamous metaplasia may be complete as in animal no. 6 treated with oestrogens for the longest time; in this animal the cornified digitations of the outer part of the cervix reached the greatest development (Pl. 1, fig. 2). But strangely enough higher up the cervical mucosa was in this case free of metaplasia whereas in other cases there was a nodular squamous metaplasia as high as, or even higher than, the fornix (nos. 3 and 4; Pl. 3, fig. 10).

As to the *endometrium of the uterine fundus*, cystic glandular hyperplasia was present in all animals though in a varying degree. The picture was like that first described by Zuckerman & Morse [1935] in the chimpanzee, but especially like that in the rhesus monkey [Zuckerman, 1937*b*]. In several of our animals glandular proliferation and cystic enlargement were very considerable. Polyps consisting of cystic glands protruded into the uterine cavity. The most outstanding polypous development was seen in animal no. 6. In this case adenomatous cystic polyps filled almost the whole enormously enlarged uterine cavity (Pl. 1, figs. 4 and 5). The epithelium of the big cysts was often flattened. There was blood in some of these cysts.

The epithelium of the uterine body was of a high cylindrical type in all the animals. But there was in one case (no. 1), besides hyperplasia of the cells of the endometrium, still another type of reaction on the part of the endometrium hitherto unknown in primates: a nodular metaplasia of the endometrium. The special arrangement of the cells, as seen in Pl. 2, fig. 7, and the difference in staining of the protoplasm compared with hyperplastic endometrium prevents any error of interpretation. It is remarkable

that *continuous* action of the oestrogen was in this animal probably only of 61 days' duration (see Table 1). There was also in two animals (nos. 1 and 4) pseudostratification of the endometrium (Pl. 2, fig. 9).

Remarkable also were the digitations of the endometrium in animal 6 (Pl. 1, fig. 5).

The *myometrium* was greatly thickened. This was due mainly to muscular hyperplasia and not, or in no considerable degree, to an increase of connective tissue. The latter was not very conspicuous even when the experiment lasted 9 months or more. But there was sometimes a considerable thickening of the uterine subserosa (no. 6). We shall return in the next section to the relevant question of the behaviour of connective tissue in our experimental animals.

There was no conspicuous increase of the *teats and mammary glands*. In most of the animals the mammary gland could not be recognized at necropsy. Neoplastic development of the mammary gland was out of the question in our five animals. Microscopical examination was made only in two cases. In one of these (no. 3) there was great development of the acinar epithelium in which vacuoles were to be seen. The lumina of the acini were filled with secretion. Acini were absent in the second case examined (no. 5). The difference between these primates and the guinea-pig in the reaction of the teats and mammary glands is very striking. The same difference is shown in rhesus [Folley, Guthkelch & Zuckerman, 1939].

#### *The behaviour of the peritoneum*

At necropsy a careful search was made for abdominal conjunctive reactions comparable to those which are so easily induced by oestrogens in guinea-pigs. In none of our animals were uterine or abdominal fibroids discovered. In one case (no. 1) two protuberances were found on the uterine wall, but microscopical examination showed them to be due to an irregularity in the disposition of the muscular layers.

Excrecences were present in another case (no. 3, 291 days) on the surface of the spleen (Pl. 4, figs. 13, 14 and 15), similar to those which have been described in our work with guinea-pigs. Though the excrecences consisted generally of sclerotic tissue only, big cells rich in protoplasm also may be found (Pl. 4, fig. 17) as is the case with the excrecences on the surface of the spleen in the guinea-pig. Likewise the characteristic hyperplastic condition of the peritoneal endothelium was present (Pl. 4, fig. 16).

A subserous cyst of the spleen also was found (no. 1) similar to those which can be observed sometimes on the kidney of the guinea-pig to which oestrogens have been administered for several months.

A fibrous thickening of the diaphragm at the limit between the muscular and membranous part was found in animal 6.

The thickening of the subserosa of the uterus which sometimes occurs has been mentioned in the preceeding section.

#### *Adenocarcinoma of the pericardium*

At necropsy of animal 6, which died after a short illness, an haemorrhagic exudate was found in the thoracic and pericardial cavities. On the top of the pericardium a tumour was present. It was hemispherical in shape and with a diameter of about



1 cm. (Pl. 5, fig. 18). The tumour was of hard consistency. The cut surface was white. On microscopical examination the tumour was shown to be composed of tubular structures lined with a cuboidal epithelium (Pl. 5, figs. 19-21). Many of these tubules were ramified. Solid epithelial strands of variable shape and size also were present (Pl. 6, fig. 22). The tubules were separated from one another by large bundles of connective fibres. A capsule of connective tissue enveloped the outer part of the tumour. The pathologist declared the tumour to be an adenocarcinoma.

A similar tumour of identical microscopical structure but much smaller and of flattened shape was found near by; this second tumour was directly connected with that described.

#### DISCUSSION

The description given above shows that with dosage and timing conditions under which abdominal fibroids were induced by oestrogens in almost every female guinea-pig, castrated or not castrated, these fibroids failed to appear in cebus. The two most important points are whether the *quantities absorbed* and the time during which there was *continuous* absorption were really comparable to those used in our guinea-pigs.

As already mentioned in the section 'Administration of oestrogens' the quantities absorbed from subcutaneously implanted tablets were in the present work with cebus several times greater than with guinea-pigs. In two animals (Table 1, nos. 1 and 6) the tablet implanted at the beginning may have been eliminated before being totally absorbed; or absorption may have greatly diminished with time (no. 6). But successively new tablets were implanted (no. 1), or injections of oily solutions of highly active esters were made (nos. 3, 5 and 6), in such a way that continuous oestrogenic action was *absolutely certain* in nos. 3, 4, 5, and 6 for *at least* 254-397 days.

Two examples may be discussed here in greater detail.

Animal 3, whose weight was about 2 kg., received the oestrogen continuously during 291 days. An average of 200  $\mu\text{g.}$  of  $\alpha$ -oestradiol was absorbed daily from the tablet. In guinea-pigs uterine and abdominal fibroids were induced with as little as 15-30  $\mu\text{g./day}$  absorbed from subcutaneously implanted tablets in the course of 80 days; the weight of these animals was 410-590 g. at the beginning and 530-600 g. at the end of the experiment [Thibaut, 1941]. But in the guinea-pig these quantities of  $\alpha$ -oestradiol are still much greater than the minimal doses that are fibromatogenic in this species. Abdominal fibroids were also induced when tablets prepared by mixing 5% of oestradiol and 95% of cholesterol were implanted subcutaneously. The same tablet weighing about 30 mg. and containing a total of 1.5 mg. of oestradiol was implanted successively in three guinea-pigs remaining in each one for 112-118 days; this tablet was able to induce tumours even in the third animal [Riesco, 1944] without the oestrogen being exhausted [unpublished work of Riesco]. The quantity absorbed in these experiments of Riesco was probably not more than 2  $\mu\text{g./day}$  and about 4  $\mu\text{g./kg.}$  of body weight per day. On the contrary, monkey 3 has absorbed during 291 days about 100  $\mu\text{g./kg./day}$ , i.e. 25 times more than the guinea-pig, without abdominal fibroids being produced. And yet more: monkey 3 received by subcutaneous injections during the last 129 days an additional quantity of 15-30  $\mu\text{g./kg./day}$  of a highly tumorigenic ester of oestradiol (17-benzoate-3-*n*-butyrate); about 7  $\mu\text{g./kg./day}$  of this ester are sufficient to induce tumours in female guinea-pigs in 90 days.

Even more striking is the example of monkey 5 with a daily absorption of about 85  $\mu\text{g.}$  of  $\alpha$ -oestradiol per kg. during 397 days and additional injections of an average of 13–26  $\mu\text{g.}$  of the 17-caprylic ester/kg./day during 240 days. A quantity of about 6  $\mu\text{g./kg.}$  of body weight per day of this ester, and often even less than that, is sufficient to induce fibroids in the female guinea-pig [Lipschutz *et al.* 1941].

The evidence that the peritoneum in cebus behaves differently from that of the guinea-pig when oestrogens are administered for a long time is overwhelming. But one might object against this conclusion that the animals dealt with in the present paper were not castrated whereas this was the case with our guinea-pigs. It has been shown in our former work that the fibromatous reaction is in the non-castrated female less pronounced than in the castrated one. But with about 12–15  $\mu\text{g.}$  of the 17-caprylic ester of oestradiol/kg./day abdominal fibroids can be elicited in most non-castrated guinea-pigs in the course of 90 days [Chaume, 1940]. Monkey 5 received twice this quantity/kg./day during 174 days preceded by 223 days of oestrogenic treatment without abdominal fibroids being elicited.

Engle *et al.* [1943] treated five rhesus monkeys with  $\alpha$ -oestradiol by implantation of tablets or injection of crystals. Though there were no exact data of the quantities absorbed these must have been very considerable; treatment lasted 24–28 months. In no animal were uterine or abdominal fibroids produced. In the work of Vargas [1943] with castrated rhesus monkeys treated with subcutaneously implanted tablets a keloid plaque was found on the fundus of the stomach in an animal killed 90 days after the total absorption of the tablet.

The appearance of an adenocarcinoma on the pericardium in monkey 6 deserves special attention. The epithelium of its tubules was so similar to that of the uterine glands and the whole picture to that of a beginning polypous formation of the endometrium (compare Pl. 5, fig. 19 with Pl. 1, fig. 5) that it was thought this tumour might be related to the uterine mucosa, i.e. that it may be considered as a metastasis of a tumour arisen from the uterine glands. But a very careful examination of the uterine mucosa revealed only cystic glandular hyperplasia not suggestive of a malignant transformation from which a metastasis might have originated. Since it was not possible to prove a relationship to the uterine mucosa one might assume that the growth originated from some cellular structures of the pericardium itself, i.e. from its lining endothelium or the subjacent cells, or from the endothelium of its lymph spaces. Our tumour reminds one somewhat of the pleural endothelioma pictured by Ewing [1942; see fig. 129 on p. 356]. Endotheliomas of the pericardium in men have also been described [McDonald, 1936; Ewing, 1942, pp. 355–6].

Two possibilities must be taken into consideration as to the origin of our neoplasm: of its being a *spontaneous* tumour due to an unknown cause, or of its being *experimentally induced* by the oestrogen administered.

Spontaneous neoplasms in primates have been reported by several authorities: Engle & Stout [1940] have lately dealt with this question. An adenocarcinoma of the rectum, in a macaque, an adenoma of the prostate in a lemur, a fibroadenoma of the uterus in a baboon, and a papilloma of the cervix in a macaque figure among these tumours. Engle & Stout add a case of a primary carcinoma of the prostate in a macaque (*Macaca mulatta*). There is no mention of a tumour similar to the present one, either in localization or in microscopical structure, occurring in primates.

As to the second possibility, it may be emphasized that in the guinea-pig the epithelium lining the lymph spaces, or small vascular spaces, of the myometrium has been seen to proliferate under the influence of a prolonged treatment with oestrogens [Lipschutz & Vargas, 1941]. But the nodules which arose under these circumstances were always composed of cells resembling fibroblasts and no tubular structures originated.

Though nothing definite can be said about the origin of this pericardial adenocarcinoma or endothelioma every one will be inclined to consider it as a spontaneous one so long as it remains a single observation under the described experimental conditions.

#### SUMMARY

Oestrogens were administered to five female New World monkeys (*Cebus*) for up to 31½ months. Continuous action of oestrogens was certain for up to 397 days and probably more.

In three out of the five monkeys absorption from subcutaneously implanted tablets of  $\alpha$ -oestradiol or of oestradiol dipropionate was coincident with the administration of highly tumorigenic esters of oestradiol (17-caprylate; 17-benzoate-3-*n*-butyrate) or of benzoestrol propionate which were given twice weekly by subcutaneous injections.

The quantities of oestrogens per kg. body weight per day administered during at least 8–13 months continuously were many times greater than those necessary to induce uterine and abdominal fibroids in female guinea-pigs in the course of 3 months only.

Uterine or abdominal fibroids developed in none of the experimental primates.

In two animals a fibrous peritoneal reaction was found, with hyperplasia of the peritoneal endothelium and cellular proliferation in one of these cases.

A high degree of cystic and polypous glandular hyperplasia of the uterine mucosa developed in all animals. There was also an insular metaplasia of the cervical mucosa. Both these types of reaction seem to be wholly similar to those described by different authorities in Old World primates.

In one animal a nodular metaplasia of the endometrium was present—hitherto not seen in the Old World primates.

There were large digitations of the cornified mucosa around the uterine opening which in one case offered macroscopically a tumorous aspect.

A tumour was discovered on the pericardium of the animal treated for the longest time. Its microscopical structure was that of an adenocarcinoma or endothelioma. As long as this tumour remains a single observation under the given experimental conditions it must be considered a spontaneous one.

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## EXPLANATION OF PLATES

## PLATE 1

- FIG. 1. Uterine opening of normal animal of 1500 g.  $\times 2$ .
- FIG. 2. Uterine opening of animal 6. Great increase and tumorous aspect. Thickening of vaginal wall.  $\times 2$ .
- FIG. 3. Frontal section of uterine cervix of no. 6. Digitations with cornified mucosa.  $\times 4$ .
- FIG. 4. Frontal section of fundus uteri of no. 6. Cysts filling almost the whole uterine cavity.  $\times 3$ .
- FIG. 5. Fundus uteri of no. 6. Cysts and polypous digitations.  $\times 56$ .

## PLATE 2

- FIG. 6. Frontal section of cervix of no. 3. Squamous epithelium alternating with hypertrophied glandular epithelium. Nodular aspect of squamous epithelial proliferation.  $\times 56$ .
- FIG. 7. Fundus uteri of animal 1. Nodular proliferation of endometrium.  $\times 110$ .
- FIG. 8. Vaginal cavity of animal 1. Detached endometrium in the vaginal cavity.  $\times 100$ .
- FIG. 9. Endometrium of animal 4. Pseudostratification.  $\times 240$ .

## PLATE 3

- FIG. 10. Frontal section of uterine cervix of animal 4. Polypous digitations. Squamous epithelium of the cervical mucosa at the level of the fornix.  $\times 12$ .
- FIG. 11. Frontal section of fundus uteri of no. 4. Cystic polypous masses filling uterine cavity.  $\times 56$ .
- FIG. 12. Same as fig. 11. Another section. Polypous masses. Detached endometrium almost in the centre of the figure.  $\times 56$ .

## PLATE 4

- FIG. 13. Spleen of animal 3. Sclerotic excrescences on the surface.  $\times 1.6$ .
- FIGS. 14, 15. Sections through spleen of no. 3. Sclerotic excrescences of different forms.  $\times 108$ .
- FIG. 16. Section through same spleen. Hyperplasia of the endothelium of the peritoneal cover near sclerotic excrescences.  $\times 480$ .
- FIG. 17. Section through excrescences of the same spleen. Big cells, partly degenerated. Sclerotic patches in the excrescences.  $\times 480$ .

## PLATE 5

- FIG. 18. Animal 6. Tumour on the apical part of pericardium.
- FIG. 19. Section through tumour. Note tubular structures separated from one another by connective tissue. Note also capsule surrounding the tumour.  $\times 47$ .
- FIG. 20. The same tumour. Tubular structure lined with epithelium whose height varies according to the tubule.  $\times 200$ .
- FIG. 21. The same tumour. Long tubule and nearby almost solid structure.  $\times 200$ .

## PLATE 6

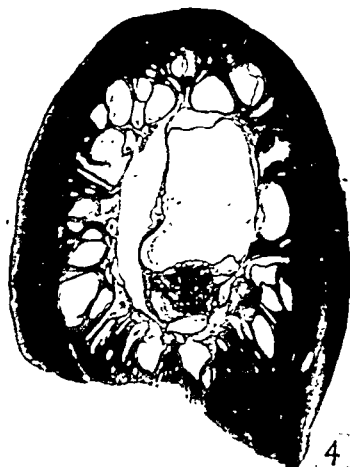
- FIG. 22. The same tumour as in Pl. 5. Epithelial cords more or less solidified.  $\times 240$ .



1



2



4

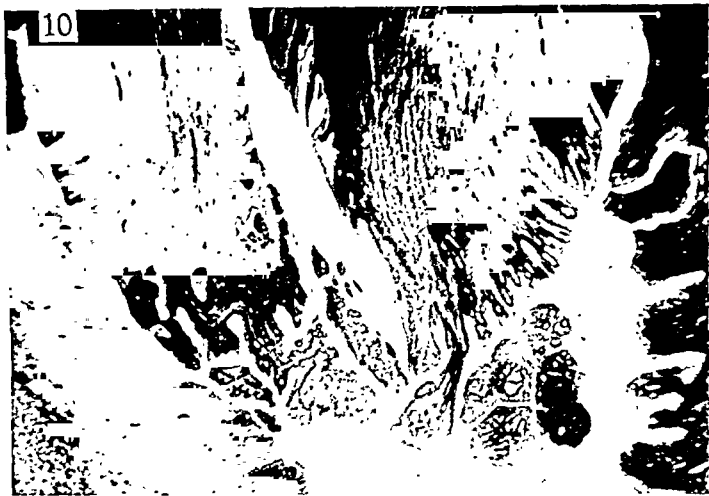


3

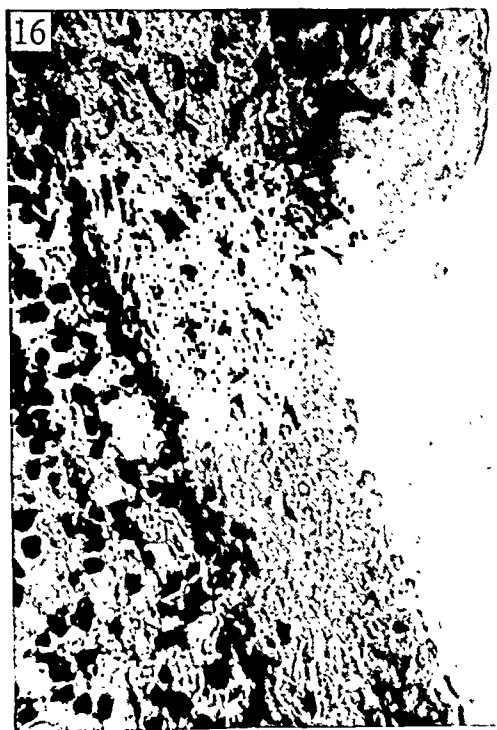


5









18



19

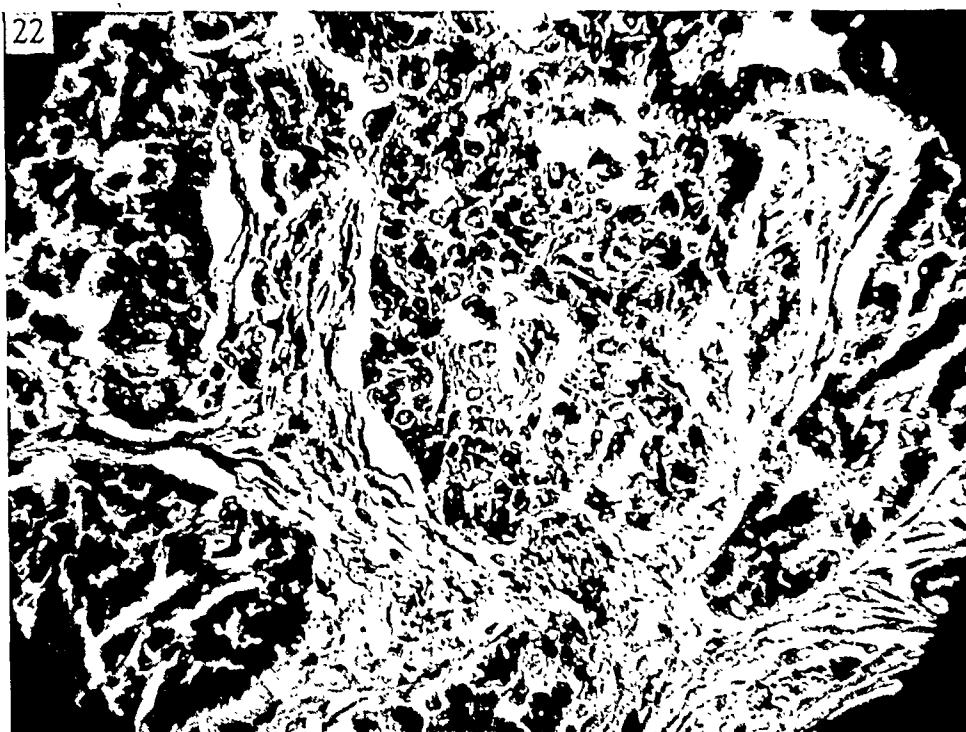


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21





# INACTIVATION OF CHORIONIC GONADOTROPHIN BY X-RAYS

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Chorionic gonadotrophin has been found to be highly resistant to the action of radium [Zondek, 1935]. The experiments with X-rays presented below show that this is true only for concentrated solutions. It should be noted that enzymes [Dale, 1940] as well as simple chemicals such as acetylcholine [Dale, 1943], and even organized elements, such as arbacia spermatozooids [Evans, Slaughter, Little & Failla, 1942], virus [Friedewald & Anderson, 1940; Lacassagne & Nyka, 1938; Luria & Exner, 1941] and erythrocytes in certain limits [Halberstaedter & Goldhaber, 1943] show a similar behaviour, that is their response to X-irradiation decreases as their concentration in the medium is increased. The effect of X-rays depends also on the constitution of the medium: it has been shown that certain materials in the medium can protect the irradiated object from the action of X-rays. It seems probable that the further study of this phenomenon could lead to a better understanding of the mechanism by which X-rays act on living cells.

## EXPERIMENTAL METHODS

A powdered preparation of chorionic gonadotrophin (Korotrin), having a potency of 100,000 i.u. (=500,000 r.u.) per gramme, was used. Though highly purified, this preparation still contains foreign matter. The gonadotrophin, weighed out on a precision balance, was dissolved in distilled water to yield a perfectly clear solution. Fresh stock solution was prepared for every assay. Samples for irradiation were mounted on hollow glass slides. A control sample was put on a glass slide and run the same way as the irradiated sample, but shielded from X-rays. The titration of irradiated and non-irradiated material was carried out (immediately after irradiation) on infantile female rats according to the Aschheim-Zondek method. Tests were carried out in 250 infantile female rats, each batch of hormone on 5 rats. Vaginal cornification and corpus luteum formation were the criteria of the test.

Irradiation was carried out by means of a dismantlable X-ray tube with copper anode at a tension of 35 kV. and a current of 15 mA. The X-ray exit was closed by an aluminium foil 30  $\mu$  thick. The irradiated solution (0.05 ml. in volume) formed a single drop in the hollowed slide. It was covered by a mica slip 0.02 mm. thick. The X-ray intensity at the distance of the drop (3.5 cm.) was about 95,000 r.p.m.

## RESULTS

### *Effect of X-rays on chorionic gonadotrophin in a dilute solution of constant concentration*

2 mg. of chorionic gonadotrophin, equivalent to 200 i.u., were dissolved in 1 ml. of distilled water and 0.05 ml. samples of this 0.2% solution were irradiated with

different doses of X-rays. Table 1 shows that a solution of chorionic gonadotrophin of this concentration is almost completely inactivated by  $6 \times 10^6$  r. Already at  $1 \times 10^6$  r. there is almost 90 % inactivation.

Table 1. *Effect of X-rays on chorionic gonadotrophin in a dilute solution of constant concentration (0.2 %)*

Dose of X-rays in million r. ...	0.1	0.3	0.5	0.6	0.7	1	6
Percentage inactivation of hormone	0	0	0	> 50 < 80	> 50 < 80	> 80 < 90	> 90

*Effect of X-rays on chorionic gonadotrophin in solutions of different concentration*

The different behaviour of chorionic gonadotrophin in diluted and concentrated forms to the action of X-rays was tested on solutions of 0.2 % (2 mg. of chorionic gonadotrophin in 1 ml. of distilled water) and 40 % (40 mg. of chorionic gonadotrophin in 0.1 ml. of distilled water) concentration. Table 2 shows that chorionic gonadotrophin is far less sensitive to the action of X-rays in the more concentrated solution.

Table 2. *Effect of X-rays on chorionic gonadotrophin in solutions of different concentration*

Dose of X-ray in million r.	Concentration of solution	Percentage inactivated
6	40 %	> 0 < 50
6	0.2 %	> 90

*Effect of X-rays on chorionic gonadotrophin in solutions containing different dilutions of serum*

2 mg. of chorionic gonadotrophin were dissolved in 1 ml. of distilled water containing human serum in the concentrations shown in Table 3. In a preliminary trial, 2 mg. of chorionic gonadotrophin were irradiated in 1 ml. of human serum. The protective action of serum on chorionic gonadotrophin is seen in Table 3, from which we deduce that as little as 0.05 % of serum completely protects chorionic gonadotrophin from the action of an X-ray dose of  $1 \times 10^6$  r. The protective action of the serum is slight but still noticeable at a serum concentration of 0.005 %.

Table 3. *Effect of X-rays ( $10^6$  r.) on chorionic gonadotrophin in 0.2 % solution containing different dilutions of serum*

Percentage concentration of serum	100	25	5	0.05	0.02	0.01	0.005	0
Percentage inactivation	0	0	0	0	> 0 < 50	> 50 < 70	> 50 < 80	> 80 < 90

In order to ascertain which part of serum affords this protection against X-rays, proteins from the serum were precipitated with acetone and desiccated. The total protein powder thus obtained was redissolved in distilled water to the concentration present in the original serum. From this stock solution dilutions of 1 : 2000 and 1 : 20,000 were prepared and chorionic gonadotrophin was added. The X-ray sensitivity of the solutions is shown by the inactivation presented in Table 4.

Table 4. *Effect of X-rays ( $10^6$  r.) on chorionic gonadotrophin in 0.2 % solution containing different concentrations of serum proteins*

Percentage concentration of serum powder solution	0.005	0.05
Percentage inactivation	> 80	0

#### DISCUSSION

The experiments show that a solution of chorionic gonadotrophin can be deprived of >90 % of its original activity by exposure to an X-ray dose of  $6 \times 10^6$  r. Inactivations of this order of magnitude are produced when the concentration of the irradiated hormone solution is low (0.2 %). In concentrated hormone solutions (40 %) inactivation of less than 50 % is elicited by doses up to  $6 \times 10^6$  r. The protection afforded in dilute solution by a very small amount of serum or serum proteins is an interesting feature.

The action of X-rays on solutes in water can be direct or indirect. Direct action implies an effect separable from the presence of solvents; indirect action implies an effect in which solvent mediation plays a major role. The direct action of radiations is elicited when the given dose of X-rays affects a constant fraction of the irradiated particles independently of their number in the irradiated volume.

Chorionic gonadotrophin responds to a given X-ray dose to a degree which depends on its concentration. It has been suggested that the explanation of this type of behaviour is that substances dissolved in water are affected by X-rays in an indirect manner. According to Dale [1942], radiation produces in water a product which in turn acts on the solute. The amount of the intermediary product depends on the amount of the solvent as well as on the X-ray dose. A given amount of the intermediary product which suffices to produce a given inactivation of a weak solution fails to produce an equal relative effect in concentrated solutions, since in the latter case the same amount of product is shared by a larger number of solute units (dilution effect). This mechanism also provides an explanation for the response observed in cases where more than one solute is present in the irradiated solution, as in the case of a solution to which serum is added. In such a solution the solutes may compete with one another for the intermediary product and the individual share of each constituent is therefore smaller (protective effect). The protective action shown by certain additions may be comprehensible on this basis.

The intermediary product, commonly called 'activated water', has apparently a transient existence. Therefore, the true nature of this state is difficult to investigate. Fricke [1934] and Loiseleur [1942] suppose it to be hydrogen peroxide in certain cases, but this assumption cannot be generalized and presents difficulties. First, irradiated water when solute is added to it after irradiation does not show the activity of water and solute which have been conjointly irradiated. Secondly, the implication that hydrogen peroxide is universally responsible for the effect of X-rays on a wide range of simple substances—chemicals (acetylcholine), enzymes, hormones, erythrocytes, spermatozooids—is unsupported. In order to meet the first objection, some authors have assumed that the observed activities are properties of hydrogen peroxide *in statu nascendi*. Even so it is difficult to understand how hydrogen peroxide can be responsible for so many varied activities.

Lea, Smith, Holmes & Markham [1944] have suggested an explanation on physical lines of the indirect action of X-rays in which the intervention of activated water is postulated. They have proved experimentally and mathematically that the ionization of the solvent by X-rays suffices to account for the indirect action of the rays in weak solution, and in particular the protection which is afforded to the solute by addition of various substances to the solution.

The direct and indirect actions of X-rays coexist in the same object (watered solutions or suspensions). When the amount of the solvent in relation to the solute is large, it seems reasonable to consider that the indirect effect is predominant as in these conditions the intermediary agent due to ionization in the water must be present in large amounts in relation to the solute. On the other hand, when the amount of solvent in relation to the solute is small, direct action due to ionization in the solute probably predominates, as in these conditions the amount of the intermediary factor is relatively small in comparison to the solute.

#### SUMMARY

1. Chorionic gonadotrophin in a low concentration (0.2%) is inactivated by the action of X-rays.

2. For a given concentration of hormone, the degree of inactivation depends on the X-ray dose. For a given X-ray dose, the degree of inactivation depends on the concentration of hormone in the irradiated solution. The same X-ray dose produces a greater percentage of inactivation at a low concentration of hormone than it does at a high concentration.

3. Addition of serum or serum proteins to the solvent (down to a concentration of 0.005%) protects the chorionic gonadotrophin from the action of X-rays.

4. This finding is explained by the assumption that the inactivation effect in dilute solutions is largely an indirect action mediated by ionized water molecules.

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# ON THE INDUCTION OF DIABETES BY MEANS OF ALLOXAN

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It was observed by Dunn, Sheehan & McLetchie [1943] that the urine of animals injected with alloxan contains a considerable amount of sugar. This phenomenon became the object of an extensive study by these and other investigators, whose results have been reviewed [Liebmann, 1944; Ingle, 1945; Chen, 1945]. A single injection of alloxan in rats, rabbits and other animals causes, to begin with, a transient but pronounced hypoglycaemia, followed in 12–48 hr. by more or less marked symptoms of diabetes, i.e. glycosuria, polyuria and high blood-sugar values. At the same time the  $\beta$ -cells of the islets of Langerhans, which are looked upon as centres for the production of insulin, are seen to degenerate. The transient attack of hypoglycaemia may be due to the release of the total amount of insulin stored in the pancreas, and this release, therefore, may be regarded as the primary effect of the injury caused by the alloxan.

Our principal object in this study has been the elucidation of the way in which this process is influenced by the hypophysis in rats. The results obtained in the initial stage of this study have already been published [Gaarenstroom & de Jongh, 1946]. A detailed account of our later experiments is given below.

## METHODS

The male and female rats used in the experiments weighed 100–160 g. and could eat and drink freely. On the 4th day of the experiment some of the animals received a subcutaneous injection of 10–25 mg. of alloxan dissolved in 0.15–0.35 ml. of water. The attack of hypoglycaemia, which should follow the injection, was prevented by administering by stomach tube 3 ml. of a 20% dextrose solution 45 min. beforehand. When the injection was ineffective, it was repeated the next day. Dose and treatment were varied somewhat according to the amount of sugar found in the urine. Four days later, i.e. on the 8th day of the experiment, the hypophysis was removed in about half the injected animals. Of the animals which had not been injected, about half were also hypophysectomized.

During four periods of 3 days each, the urine excreted by the animals which had been injected with alloxan was collected, and its amount and sugar and nitrogen contents determined: sugar by the titration method according to Benedict, and nitrogen by means of the micro-Kjeldahl method. The 3-day periods extended from the 1st to the 3rd, from the 6th to the 8th, from the 9th to the 11th, and from the 12th to the 14th days: the first set of estimations, therefore, was made before the alloxan injection, the second after the latter but before hypophysectomy, and the third and fourth after this operation. In addition, on the 8th and 14th days blood-sugar values were determined before and after the administration of a 20% dextrose



solution. The latter was given as an oral dose of 3 ml. 2 hr., together with an intra-peritoneal injection of 2 ml. administered 1 hr., before the determination of the blood-sugar value. The blood-sugar values were determined by the Hagedorn-Jensen method. The changes in body weight too were noted, and in some of the animals the amount of acetone in the urine was estimated.

Of the hypophysectomized control animals, i.e. those that did not receive an alloxan injection, the urine was collected during two periods of 3 days in order to determine its nitrogen content. These figures served for comparison with the corresponding ones obtained from rats injected with alloxan. Finally blood-sugar values were determined before and after the administration of dextrose.

Of the non-hypophysectomized control animals too the blood-sugar value was estimated, but no attempt was made to estimate the nitrogen content of the urine, as the latter would not have differed from that found in the urine of the test animals in the period before the alloxan injection.

Chemical estimations were not considered reliable unless duplicate values obtained from two different samples were found to agree. The difference between the duplicate values rarely exceeded 10 % in case of the urinary dextrose determinations, 10 mg. per 100 ml. in case of the blood-sugar estimations, and 20 mg. in case of the nitrogen determinations. If they did, the determinations were rejected. The efficacy of the hypophysectomy was tested by histological examination of the sella turcica.

## RESULTS

### *General*

The administration of alloxan and the hypophysectomy caused a number of casualties: the mortality due to the injection varying between 10 and 20 %, and that caused by the gland extirpation between 30 and 40 % of the animals. Data obtained from animals succumbing before the 12th day of the experiment were rejected.

About 50 % of the animals to which alloxan was administered reacted with either no sugar-excretion or with very little. Those that excreted in the second period (as stated above, alloxan was administered in the interval between the first and the second periods) 2 g. of sugar or less were placed in a separate group. In this way six groups were formed: (1) animals injected with alloxan excreting more than 2 g. of sugar; (2) similarly treated animals excreting less than 2 g.; (3) animals that were not injected; (4) injected and hypophysectomized animals excreting more than 2 g.; (5) similarly treated animals excreting less than 2 g.; and (6) hypophysectomized animals that had not been injected.

Each group consisted of 8-16 rats. The results are summarized in Table 1. To save space the figures are not given in full but are represented by their mean values and ranges.

### *Sugar excretion in the urine*

Rats that have not been treated with alloxan do not excrete dextrose, or, if they do, the amounts are negligible. In those cases where the amounts have been determined, they are, therefore, not mentioned in the table. After the administration of alloxan the rats that reacted excreted on the average 1.5-2 g. of sugar daily (4.5-6 g. in a period). One rat excreted as much as 3-4 g. daily. The sugar excretion reached its maximum in the second period, after which it tended to decrease. The group of rats

Table 1

		Non-hypophysectomized rats				Hypophysectomized rats			
		Alloxan injected			No treatment	Alloxan injected			No treatment
		High sugar excretion group*	Low sugar excretion group*			High sugar excretion group*	Low sugar excretion group*		
No. of animals	...	11	11		16	11	8		9
Body weight (g.):									
1st day		124 (99-152)	137 (120-154)		—	126 (113-148)	137 (128-150)		—
4th day		—	134 (118-149)		—	122 (102-140)	—		—
8th day		108 (81-126)	120 (107-134)		—	105 (88-130)	124 (103-142)		—
11th day		115 (88-137)	124 (102-144)		—	111 (95-132)	—		—
14th day		101 (70-118)	121 (112-134)		132 (104-167)	103 (80-125)	114 (104-126)		129 (121-138)
Amount of urine (ml.):									
1st period		8 (4-11)	12 (7-23)		—	10 (4-15)	17 (7-27)		—
2nd period		65 (32-134)	40 (25-64)		—	88 (48-133)	36 (18-67)		—
3rd period		88 (40-156)	36 (21-80)		—	92 (49-140)	62 (20-117)		70 (38-112)
4th period		78 (21-156)	29 (10-66)		—	55 (18-72)	49 (21-108)		44 (22-63)
Sugar in urine (g.):									
2nd period		4.5 (2.0-11.0)	0.6 (0.0-1.6)		—	5.3 (2.1-8.3)	0.4 (0.0-1.5)		—
3rd period		6.1 (2.0-10.4)	0.8 (0.0-3.1)		—	1.3 (0.0-5.7)	0.0 (0.0-0.2)		—
4th period		5.4 (0.6-8.2)	0.3 (0.0-2.6)		—	0.6 (0.0-2.7)	0.0		—
Blood-sugar values (mg. per 100 ml.):									
5th day: Fasting		98 (62-145)†	94 (64-123)		—	194 (90-378)	104 (90-148)		—
After glucose		348 (330-375)	377 (238-540)		—	421 (346-565)	356 (150-567)		—
14th day: Fasting		116 (60-226)	92 (69-118)		74 (54-83)	49 (40-59)	52 (40-58)		52 (41-65)
After glucose		350 (247-490)	273 (173-427)		116 (86-182)	387 (277-528)	321 (167-536)		170 (102-358)
Nitrogen in urine (mg.):									
1st period		208 (112-294)	266 (134-375)		—	270 (132-352)	358 (140-479)		—
2nd period		519 (336-908)	354 (173-464)		—	518 (246-800)	402 (263-623)		—
3rd period		595 (411-1000)	494 (274-813)		—	526 (420-659)	395 (213-505)		426 (335-497)
4th period		510 (137-783)	386 (206-472)		—	424 (262-614)	373 (185-539)		500 (385-681)

\* During 1st period.

† Estimated in only 4 animals.

that did not react, i.e. those that during the first period did not lose more than 2 g. of sugar, showed a similar behaviour, although the top of the curve in this case was, of course, much lower.

When the animals which had been treated with alloxan were hypophysectomized, the sugar excretion showed a sharp fall. Of the 9 rats which in the period before the extirpation together had excreted 45.2 g. of dextrose and were still alive at the end of the experiment, three only continued to excrete sugar in the '4th period', and the total amount produced in the latter was 5.1 g. only. In the animals which had reacted weakly to the alloxan injection, the sugar excretion almost immediately fell to zero after removal of the pituitary.

#### *Blood-sugar values*

In the animals injected with alloxan the fasting blood-sugar value appeared to be increased: whereas in normal rats an average value of 74 mg. per 100 ml. was found, the mean values observed in the different groups of injected rats were respectively 92, 94, 98, 104, 116 and 194 mg. per 100 ml. In the injected rats which showed no high sugar excretion, the values were on the whole lower but the difference was small. In the injected rats removal of the hypophysis led to very low values, namely 49 and 52 mg. per 100 ml. In the hypophysectomized control animals a similar value was found: 52 mg. per 100 ml.

After the administration of sugar the blood-sugar level increased as a rule to values of 250 mg. per 100 ml. or more.\* It should be noted that this increase was independent of the presence of the hypophysis. In the injected animals which showed a high sugar excretion, the blood-sugar value was somewhat higher than in the injected animals which reacted with low excretion, but the difference was not considerable. In all the injected animals the blood-sugar values were markedly higher than in the animals which had received no alloxan. Among the latter the normal rats showed an increase which lagged behind that observed in hypophysectomized ones.

#### *Amount of urine*

In the animals which had been injected with alloxan, the amount of urine showed a marked increase: it was, in fact, sometimes more than ten times as large as before the injection. There is, therefore, some relation between the increase of the amount of urine and the excretion of sugar, but certainly there is no proportionality between the two. The animals which reacted weakly to the alloxan, and even those that excreted no sugar at all, showed a fairly strong polyuria. The removal of the hypophysis had hardly any effect on the amount of urine excreted by animals that reacted positively to the alloxan injection, whereas in animals that reacted weakly or not at all, an increase was noted. In the hypophysectomized animals which had received no alloxan, the amounts were nearly the same.

#### *Excretion of nitrogen*

The administration of alloxan markedly increased the nitrogen content of the urine. This appeared to be a general rule. The increase of the nitrogen value in the rats which reacted with a high excretion of sugar amounted on the average to 265%

\* In our earlier experiments, of which the results have been published elsewhere, a smaller amount of sugar was administered, and the increase of the blood-sugar level accordingly was less striking.

and to 184% in those that reacted weakly or not at all. A comparison between the nitrogen content of urine excreted by the control animals after extirpation of the hypophysis, with that of the urine excreted by the test animals before they had been injected, shows that hypophysectomy too causes an increase. As alloxan injection and hypophysectomy affect the nitrogen excretion in a similar way, it will be difficult to decide what part of the increase is to be assigned to each of them when they are acting simultaneously.

#### *Body weight*

After the administration of alloxan the body weight showed, at least in the beginning, a decrease. Between the latter and the extent of the sugar excretion, there appeared to be a certain parallelism. Hypophysectomy affected the body weight but little.

#### *Ketosis*

Acetone was never found in the urine of rats injected with alloxan.

### DISCUSSION

The results of our experiments confirm and extend those obtained by the investigators of other countries in their study of the diabetes induced by alloxan. They are also of importance for our knowledge of the patho-physiology of diabetes in general.

In the first place our experiments allow us to draw some conclusions with regard to the cause of the increased nitrogen excretion in diabetes. This increase, which in the figures given above is clearly perceptible, must be the result of an intensified protein decomposition but this may be attributed to various causes. The most plausible supposition would be that the decrease in the consumption of carbohydrates makes it impossible to obtain the required amount of energy in the ordinary way, and therefore necessitates the decomposition of other substances, such as proteins and fat. It is also possible that insulin favours the synthesis of proteins, and that the latter therefore decreases because of the damage done to the pancreas. Finally, the so-called glyconeogenesis from proteins, i.e. the transformation of proteins into carbohydrates might, in case of an insulin deficit, be intensified. All three possibilities have been advocated [cf. the reviews by Esveld, 1942; Marx & Evans, 1944; and Lukens, 1944]. None of these possibilities is actually excluded by our experiments though, as even in those animals that excreted little or no sugar the nitrogen excretion was markedly increased, the latter cannot be due to low level of carbohydrate consumption alone; it must be assumed that the insulin deficit acts in another way also, either as a check to protein synthesis or as an accelerator of the transformation of proteins into carbohydrates.

Our experiments also shed some light on the decrease which the sugar excretion of diabetic animals undergoes when the hypophysis is removed. This phenomenon, which in our experiments too was clearly perceptible, was first noted in 1930 [Hous-say, 1942]. Though several hypotheses for the elucidation of this effect have been brought forward in which a number of hormones produced by the hypophysis and acting on the carbohydrate metabolism are suggested as playing a part, no fully satisfactory explanation has as yet been given. Owing to technical difficulties the various possibilities could not be tested. Pancreas extirpation could not be carried out satis-

factorily in animals of a smaller size than cats or dogs, and the latter were not available in sufficient numbers. Moreover, hypophysectomy is in these animals very difficult, and the effects of both operations are so serious that it is difficult to keep the animals alive. The induction of diabetes by means of alloxan, therefore, is an important acquisition, for it allows the use of small animals, which are more resistant and more easily hypophysectomized, and of which larger numbers are available, so that serial experiments can be carried out.

The decrease in sugar excretion after hypophysectomy might be ascribed to various causes. The most plausible ones are:

- (1) that less food is taken, and therefore less sugar absorbed;
- (2) that the consumption of sugar in the body, which is greatly reduced after the alloxan injection, resumes its former value;
- (3) that the transformation of protein, and perhaps that of fat too, into carbohydrates, which may have been enhanced by the alloxan treatment, undergoes a decrease.

The first cause certainly cannot be neglected. The hypophysectomized animals eat, as a rule, but little, and the amount of carbohydrates which they absorb is accordingly small. This must, of course, affect the dextrose excretion. Experiments in which the food consumption of hypophysectomized and non-hypophysectomized animals injected with alloxan is being studied are at present under way. Apart from the fact that the animals eat less, we have to consider the possibility that less sugar enters the circulation because the absorption from the intestinal tract is unfavourably affected by the removal of the hypophysis. The reduction of the amount of carbohydrates which the animals absorb, however, cannot be the sole cause of the decrease in sugar excretion. The fact that blood-sugar values which were determined when the injected animals had fasted 16 hr. or longer (and so may be regarded as nearly independent of the amount of food previously consumed) proved to be considerably increased in non-hypophysectomized animals and distinctly decreased when the hypophysis was removed, suggests the presence of an endogenous cause in addition to the reduction of the amount of sugar absorbed by the animals.

That the consumption of sugar in the body should be accelerated after hypophysectomy has often been claimed [cf. Russell, 1938, 1942, 1943], but this assumption finds no support in our experiments. The very high values of the blood-sugar level observed in hypophysectomized alloxan-treated animals after the administration of sugar are a strong argument against this view. That this increase does not lag behind the one observed in non-hypophysectomized animals injected with alloxan proves that the removal of large amounts of sugar suddenly brought into the circulation is in both cases a very difficult task. In rats that are neither injected nor hypophysectomized, the increase is much smaller.

As the second cause can be excluded, we must assume that the decrease of sugar excretion after hypophysectomy is due at least in part to the third: a decrease of the rate with which proteins are transformed into carbohydrates. This view has been advocated already by others, and various arguments in favour of it have been collected [cf. Long, 1943; Marx & Evans, 1944]. Until now, however, the possibility that the decrease in the sugar excretion might become entirely due to the two other causes has never been excluded.

Another phenomenon which deserves our attention is the decrease in sugar excretion during the course of the experiment. In this we might see a tendency to recover. The presence of such a tendency showed itself clearly in four rats of which we studied the dextrose excretion during two periods of 3 days, namely, immediately after the alloxan injection and 20 days later: during the first period the total amount appeared to be 13.9 g., whereas in the second it was 1.7 g. only, a decrease of nearly 90 %. This clearly demonstrates the tendency of the injured pancreas to recover spontaneously, which is the more remarkable as in cases of spontaneous diabetes in man the tendency is in the opposite direction. Should this mean that the injurious agent in man, in contrast to the agent in our experiments, remains constantly active? In that case it would be appropriate to look for this agent with the utmost energy. This explanation, however, is an improbable one, as a permanent diabetes can be induced by a short series of injections with a diabetogenic pituitary extract. Presumably the injury caused by the alloxan injection is of a less irreversible character than that which in human pathology causes diabetes.

The polyuria observed in rats injected with alloxan appeared to be only loosely connected with the excretion of dextrose, for even in animals which excreted no sugar the amount of urine was increased though to a lesser extent. So the diabetic polyuria may be not merely a consequence of the sugar excretion but due to an injurious effect of alloxan on the kidneys. According to Dunn *et al.* [1943], after administration of alloxan an injury in the kidneys was indeed revealed by histological examination, but it is uncertain whether this injury is responsible for the polyuria. The renal function does not seem to be impaired too seriously in our rats as the excretion of nitrogen had increased. That the disappearance of the polyuria coincides with a decrease in the sugar excretion, might be regarded as an indication of the presence of a relation of some kind with the activity of the pancreas. This problem will have to be studied more closely.

The character of the diabetes induced in our experiments by the administration of alloxan cannot be called severe. As the amount of food which the animals ate daily was not determined, we are unable to say how much carbohydrate they still consumed. The loss in body weight was only moderate and was, moreover, restricted to the first few days after the alloxan injection; the fasting blood-sugar values were not exceedingly high, and acetone was never found in the urine. However, it should not be forgotten that the animals which died shortly after the alloxan administration were not taken into account and may have had a diabetes of a more serious character. In the literature dealing with diabetes induced by alloxan cases with lethal effect have been often reported.

#### SUMMARY

Subcutaneous administration of alloxan (one or two injections of 100–200 mg. per kg. body weight) causes in about half the number of injected rats a considerable excretion of sugar. The fasting blood-sugar level rises above that of normal rats, and after giving dextrose orally and intraperitoneally, the increase of the blood-sugar values is very great. A polyuria and an increase in the nitrogen content of the urine are manifested at the same time.

In those rats that do not react to the alloxan injection by the excretion of considerable amounts of sugar, the blood-sugar level nevertheless shows similar deviations

from the normal, and in these animals too the amount of urine and its nitrogen content are increased.

The extirpation of the hypophysis causes a marked decrease or even the total disappearance of the sugar excretion. The fasting blood-sugar level sinks below that of normal rats, and is not more than that of hypophysectomized rats which did not receive alloxan. After the administration of sugar, however, the blood-sugar level of hypophysectomized rats injected with alloxan reaches a very high value, as high as that of non-hypophysectomized rats treated in the same way. The polyuria and the increased nitrogen content of the urine persist after the extirpation of the hypophysis, but values of the same order of magnitude were found in hypophysectomized control rats.

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# FATE OF SOME OESTROGENIC PHENANTHRENE AND STILBENE DERIVATIVES IN THE RAT

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It is well established that many oestrogens are less active when given by mouth than when administered parenterally in the same dose, while other oestrogens have much the same activity by either route. From consideration of the current literature [Kemp & Pedersen-Bjergaard, 1943] it appears that the natural oestrogens belong to the first group and the synthetic oestrogens developed by Dodds, Golberg, Lawson & Robinson [1938, 1939] to the second.

In accordance with this, Biskind & Mark [1939] found that pellets of oestrone implanted in the portal field did not produce vaginal oestrus, whereas Allen [1941] *did* produce oestrus with similarly placed stilboestrol pellets. Allen concluded that stilboestrol, unlike oestrone, is not inactivated in the liver.

## METHODS OF ASSAY

All the assays recorded in the tables were carried out on adult spayed rats which had received a fortnight previously a priming dose of  $3\mu\text{g.}$  of oestrone in 0.2 ml. of olive oil. All the test substances were given in aqueous solution or suspension and the doses recorded are those producing complete vaginal cornification in 50% of the animals ( $\text{ED}_{50}$ ). This dose has been found in the usual way by means of a dose:response curve and calculated by the method proposed by Pedersen-Bjergaard [1939], with the standard error expressed in percentage terms.

When the dose was given in divided lots it was given in 5 parts during 50 hr., otherwise it was given as a single administration.

## EXPERIMENTAL

### *Direct comparison of oral and parenteral activity*

The results given in Table 1 indicate that the division between the natural and synthetic oestrogens in the ratio of oral:subcutaneous dose is not a clear one. Among the synthetic oestrogens hexoestrol has a comparatively large ratio, while oestriol has a ratio of almost unity among the natural oestrogens. The results with hexoestrol do not accord with those of Campbell, Dodds, Lawson & Noble [1939] who reported an oral:parenteral ratio of about unity with this compound as with stilboestrol and dienooestrol.

### *Comparison of activities by intravenous injection*

In this experiment the six substances were injected by single administration directly into the femoral vein and into a small branch of the portal vein. The method adopted has been described by Pedersen-Bjergaard [1939] but was here modified to the extent that the total dose (4 ml.) given by continuous infusion during 20 min. was administered by means of a specially constructed apparatus. The results are recorded in Table 2.



Table 1. *Relative oral and subcutaneous activities of six oestrogens given to spayed rats (five doses in aqueous solution or suspension during 50 hr.)*

Oestrogen	Subcutaneous administration		Oral administration		ED <sub>50</sub> ratio: oral subcutaneous
	No. of rats	ED <sub>50</sub> μg. ± S.E.	No. of rats	ED <sub>50</sub> μg. ± S.E.	
Synthetic oestrogens					
Hexoestrol	60	0.50 ± 10%	60	40 ± 20%	80
Stilboestrol	60	0.40 ± 10%	60	4.0 ± 20%	10.
Dienoestrol	60	1.30 ± 10%	60	3.8 ± 30%	3
Natural oestrogens					
Oestriol	60	18 ± 10%	60	25 ± 20%	1.4
Oestradiol	60	0.15 ± 7%	60	90 ± 20%	600
Oestrone	60	1.11 ± 7%	60	220 ± 20%	200

Table 2. *Relative oestrogenic activities of six oestrogens in spayed rats when administered intravenously into a branch of the portal vein or into the femoral vein (single dose in 4 ml. of aqueous solution or suspension given during 20 min.)*

	Femoral administration		Portal administration		ED <sub>50</sub> ratio: portal femoral
	No. of rats	ED <sub>50</sub> μg. ± S.E.	No. of rats	ED <sub>50</sub> μg. ± S.E.	
Synthetic oestrogens					
Hexoestrol	60	267 ± 15 %	60	333 ± 20 %	1.2
Stilboestrol	60	225 ± 15 %	60	275 ± 20 %	1.2
Dienoestrol	60	30 ± 15 %	60	15 ± 20 %	0.5
Natural oestrogens					
Oestriol	20	750 ± 20 %	20	750 ± 30 %	1
Oestradiol	60	150 ± 15 %	60	500 ± 20 %	3.3
Oestrone	60	250 ± 15 %	60	2500 ± 20 %	10

The results in Table 2 agree on the whole with those in Table 1. The ratio of activities by the two routes is here again low for stilboestrol, dienoestrol, and oestriol indicating that these substances are inactivated little or not at all in the liver, while oestradiol and oestrone on the other hand have relatively high ratios indicating hepatic inactivation. The results with hexoestrol, however, are not concordant in the two experiments—the results of the intravenous injections do not show any evidence of liver inactivation such as was found in the previous experiment.

#### *Further experiments with hexoestrol*

In an effort to explain the discrepant result we carried out an experiment to determine whether any large proportion of hexoestrol was not being absorbed and so was eliminated in the faeces, but even after the oral administration of as much as 1 mg. per rat we were unable to recover more than 10% in the faeces, a proportion we could also recover when the same dose of stilboestrol or dienoestrol was given.

*Method.* The faeces were collected during 24 hr. and carefully ground with pyridine and left standing overnight. The pyridine extract was filtered off, the faeces re-extracted and the combined pyridine extracts evaporated. The residue was dissolved in a small volume of ethanol and assayed for oestrogenic activity in spayed mice after dilution with water.

Further experiments both *in vitro* and *in vivo* failed to demonstrate any inactivation of hexoestrol by the contents of the stomach or small or large intestines. We therefore concluded that inactivation of hexoestrol must be taking place in the liver but that the intravenous injection method failed to demonstrate it.

#### *Comparisons in rats with liver damage*

Using the method devised by Talbot [1939] and Pincus & Martin [1940], we intoxicated some of our test rats with carbon tetrachloride in order to depress liver function so that oestrogen inactivation should be depressed also for as long as the intoxication lasted.

*Method.* The spayed rats were intoxicated by being given a well-shaken mixture of 0.1 ml. of carbon tetrachloride, 0.1 ml. of ethanol, and 0.1 ml. of water each morning for 4 days by stomach tube. The first dose of oestrogen was given 3 hr. after the first dose of the carbon tetrachloride mixture; four subsequent doses of oestrogen were given at roughly 12 hr. intervals.

The results in Table 3 show that under these conditions the ratios of oral to parenteral activity are much alike for all six compounds, which indicates that oestrone, oestradiol, and hexoestrol are inactivated in the liver although the intravenous experiment for some reason failed to show this in the case of hexoestrol.

Table 3. *Relative oral and subcutaneous activities of six oestrogens given to spayed rats with liver damage (five doses in aqueous solution during 50 hr.)*

Oestrogen	Subcutaneous administration		Oral administration		ED <sub>50</sub> ratio: oral subcutaneous
	No. of rats	ED <sub>50</sub> μg. ± S.E.	No. of rats	ED <sub>50</sub> μg. ± S.E.	
Synthetic oestrogens					
Hexoestrol	60	0.30 ± 30 %	100	3.0 ± 40 %	10
Stilboestrol	60	0.30 ± 20 %	60	3.2 ± 20 %	11
Dienoestrol	60	1.10 ± 20 %	60	3.2 ± 30 %	3
Natural oestrogens					
Oestriol	20	0.50 ± 50 %	20	0.25 ± 50 %	0.5
Oestradiol	30	0.16 ± 20 %	40	0.50 ± 40 %	3
Oestrone	30	1.20 ± 20 %	60	3.00 ± 40 %	2.5

One peculiar feature of the results is that while the other oestrogens show almost the same activity by subcutaneous injection in the normal and intoxicated rats, oestriol does not. The effective dose of oestriol both orally and parenterally is reduced, 36-fold and 100-fold respectively. We must conclude that this compound is inactivated in the liver whether given orally or subcutaneously and almost to the same degree.

#### DISCUSSION

On the basis of these results we may separate the six oestrogens we have investigated into three groups.

(1) Those oestrogens which are not inactivated by the liver, whether they are given orally or parenterally: stilboestrol and dienioestrol.

(2) Those oestrogens which are inactivated in the liver when given orally: oestrone, oestradiol, and hexoestrol.

(3) Those oestrogens that are inactivated by the liver whether given orally or parenterally: oestriol.

In this classification the differences are relative, not absolute: there may be some slight inactivation of stilboestrol and dienioestrol in the liver, while the inactivation of oestrone, oestradiol, hexoestrol, and oestriol is not complete under the conditions of the experiments.

On the whole our results have been concordant, apart from the case of hexoestrol where the results of the intravenous experiment did not agree with those of the liver intoxication experiment. The explanation of this may lie in the different time of the dosages in the two cases. In the intravenous administration the operative procedure for exposing the portal vein is severe so that the dose has to be given within an hour and cannot be repeated. In the rats with liver damage the dosage could be spread over 50 hr.—a period more like that during which the oestrogens act in the normal oestrous cycle in the rat.

Differing techniques, methods of administration or investigation, strains of rat, species of animal, and solutes may easily give rise to differing quantitative results in oestrogen investigation. This is so with our results, which for example differ from those of Odell, Skill & Marrian [1937] as to the relative activities of oestriol. Zondek, Sulman & Sklow [1943] found that liver inactivated stilboestrol *in vitro*, though not to so great an extent as oestrone. Segaloff [1944] also finds that stilboestrol is inactivated in the liver but again oestrone, oestradiol, and hexoestrol are more rapidly inactivated: these results were obtained using intrasplenic injections or implantations. These findings do not appear to us contradictory of our own. As we have pointed out above the differences we have found may be quantitative only; the only conclusion we wish to draw is that the differences are sufficiently great to allow no doubt which group each oestrogen of the six belongs to when considering their oral and parenteral activity.

#### SUMMARY

The oral and subcutaneous activities of stilboestrol, dienioestrol, hexoestrol, oestrone, oestradiol, and oestriol have been compared in spayed rats and in spayed rats with liver damage produced by intoxication with carbon tetrachloride.

The results indicate that stilboestrol and dienioestrol are not inactivated in the liver when given by mouth, while oestrone, oestradiol, and hexoestrol are. Oestriol is apparently inactivated by the liver whether given orally or parenterally.

Comparisons by intravenous injection into the portal vein and femoral vein broadly supported these conclusions with certain anomalies which are discussed.

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# FAILURE OF LACTOGENIC HORMONE TO MAINTAIN PREGNANCY INVOLUTION OF THE THYMUS

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In recent papers [Grégoire, 1946, 1947] the factors involved in the maintenance of pregnancy involution of the thymus during suckling were studied, and it was concluded that endocrine, and not metabolic, factors were probably primarily concerned. It was suggested that variations in the secretion of anterior pituitary hormones (prolactin, adrenocorticotrophin) occurred under the influence of variations in suckling stimuli or milk removal, and that these changes prevented thymus regeneration in different degrees either directly or by altering the secretion of thymus-depressing hormones.

The experiment reported here aimed to determine whether the injection of physiological doses of lactogenic hormone would prevent the regeneration of the thymus that takes place in females whose young are removed immediately after parturition. The few inconclusive reports of the influence of lactogenic hormone on the thymus and lymphoid tissue were reviewed in the earlier paper [Grégoire, 1947].

## METHODS

### *Animals*

Thirty-seven albino female rats of our strain were used. All of them were primiparous and 6-7 months old. Their litters were removed at birth and the mothers were spayed—thus reproducing the conditions of the earlier experiments where it was shown that ovariectomy had no effect on the thymus reactions during lactation.

### *Injections*

The pituitary lactogenic extract used (Physolactin, Glaxo) contained 12 Riddle units per mg. It was dissolved in *N*/500 NaOH and adjusted to pH 10 with *N*/10 NaOH so that the final concentration was 10 mg./ml. Twenty of the rats were injected subcutaneously with 0.3 ml. of this solution daily for 10 days, thus receiving total doses of 360 Riddle units each. Seventeen rats were injected with the same volume of solvent at pH 10 to serve as controls.

### *Autopsy*

The animals were killed 24 hr. after the last injection (11th day after parturition). Thymus, thyroid glands, adrenals, spleen and various lymph-nodes (axillary, subscapular, inguinal groups of both sides, and the two superior iliac or lumbar lymph-nodes) were removed and weighed on a torsion balance after fixation in Bouin-Hollande-sublimite. Portions of the mammary glands from different sites (particularly the inguinal glands) were fixed for histological study.

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## RESULTS

*Organ weights*

Average results are recorded in Table 1. For reasons given in the previous paper [Grégoire, 1947] relative weights (expressed as g./kg. body weight) have been chiefly used, and those animals that maintained or gained weight (subgroups A) are separated from those (subgroups B) that lost weight.

The statistical significance of any difference has been established by calculation of the probable error of the difference of the means—probable errors are included in the Table in borderline cases only. The slight variations in body weight are statistically insignificant or merely reflect daily fluctuations that are usual in animals of this age.

There is no significant difference between the thymus weight in control and experimental groups. Regeneration has evidently been active in all animals, the thymus weights being about double that found immediately after parturition in females of the same age and body weight.

The weights of the spleen and of the superficial lymph-nodes are significantly higher in the experimental animals; the difference in the weights of the iliac lymph-nodes is not significant. The difference in spleen weight is not so significant when all the animals are considered as when only those with weight maintenance are considered (A subgroups).

There is no difference in adrenal-gland weights and the thyroid differences are only doubtfully significant ( $P = 0.2$  in the A subgroups and  $0.11$  in the total (A + B) groups).

*Histology*

The prolactin injections had apparently no effect on the histology of the thymus.

There was hypertrophy of the cortical fundamental tissue of the superficial lymph-nodes in the experimental animals, which also showed irregular increase in the size of the germinal centres. Pycnotic lymphocyte nuclei were found scattered or congregated in phagocytes. No measurements of the amount of white (lymphoid) pulp in the spleen were made.

The thyroid glands of most of the animals were almost completely inactive, with low follicular height, colloid storage, and scarcity of absorption vacuoles. At parturition the thyroid gland is usually hyperactive. When epithelial height measurements were made in 100 follicles of seventeen experimental and seventeen control animals, the height was found to be significantly lower in the injected animals (Table 1 and Fig. 1). Histologically the glands of the prolactin-injected rats appeared more inactive than the controls.

The mammary glands of the control rats were completely involuted, the lobules being reduced to a few islands of collapsed alveoli and ducts scattered in adipose tissue. In a few cases the regression was less marked and lumina might be found in some alveoli but they did not contain any secretion.

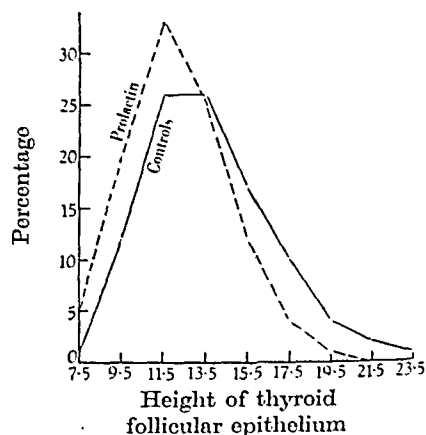


FIG. 1. Frequency distribution curves of the follicular-epithelial heights in thyroid glands of control and prolactin-injected animals (camera lucida drawings:  $10\text{mm.} \equiv 5\mu$ ).

Table 1. Average data in the seventeen control rats and the twenty prolactin-injected rats; number of animals in the A and B subgroups are given in parenthesis

number of animals in the 11 days after delivery

	Group I. Spayed and weaned females (17 controls) 11 days after delivery						Group II. Spayed, weaned and prolactin-injected females (20) 11 days after delivery											
	A (10)			B (7)			A+B			A (8)			B (12)			A+B		
	Av.	P.E.		Av.	P.E.		Av.	P.E.		Av.	P.E.		Av.	P.E.		Av.	P.E.	
Age at delivery (days)	202	—		212	—		206	—		197	—		201	—		199	—	
Body wt. (g.):																		
At delivery	201	—		192	—		198	—		197	—		201	—		199	—	
When killed	206	—		186	—		198	—		200	—		192	—		196	—	
	+2.8	±0.63		-3.5	—		-0.2	—		+1.8	±0.52		-4.0	—		-1.7	—	
Varia tions in body wt. since delivery (%)																		
Wt. of thymus:																		
Actual (g.)	0.2848	—		0.2392	—		0.2661	—		0.2935	—		0.2497	—		0.2672	—	
Relative (g./kg.)	1.389	±0.090		1.286	—		1.347	±0.060		1.454	±0.070		1.302	—		1.363	±0.0414	
Wt. of thyroid gland:																		
Actual (g.)	0.0162	—		0.0158	—		0.0161	—		0.0141	—		0.0146	—		0.0145	—	
Relative (g./kg.)	0.078	±0.0033		0.085	—		0.081	±0.0023		0.071	±0.0033		0.076	—		0.074	±0.0019	
	—	—		—	—		13.6	±0.30		—	—		—	—		12.3	±0.21	
Ht. of thyroid epithelium (mm. in drawing)																		
Wt. of adrenals:																		
Actual (g.)	0.0509	—		0.0531	—		0.0518	—		0.0532	—		0.0543	—		0.0539	—	
Relative (g./kg.)	0.246	±0.0052		0.296	—		0.263	—		0.261	±0.0100		0.282	—		0.274	—	
Wt. of spleen:																		
Actual (g.)	0.449	—		0.476	—		0.461	—		0.595	—		0.468	—		0.519	—	
Relative (g./kg.)	2.17	±0.09		2.56	—		2.33	±0.10		3.00	±0.20		2.39	—		2.64	±0.12	
Wt. of lymph nodes:																		
Sub-lingual and axillary groups:																		
Actual (g.)	0.104	—		0.110	—		0.106	—		0.158	—		0.142	—		0.148	—	
Relative (g./kg.)	0.508	—		0.581	—		0.535	±0.021		0.795	—		0.734	—		0.757	±0.039	
Inguinal groups:																		
Actual (g.)	0.055	—		0.065	—		0.059	—		0.071	—		0.072	—		0.072	—	
Relative (g./kg.)	0.272	—		0.345	—		0.299	±0.015		0.360	—		0.368	—		0.365	±0.024	
Pit. group:																		
Actual (g.)	0.030	—		0.039	—		0.033	—		0.030	—		0.031	—		0.031	—	
Relative (g./kg.)	0.145	—		0.206	—		0.198	—		0.151	—		0.163	—		0.159	—	

In the prolactin-injected females this involution of the mammary gland was delayed in all but one case. The islands of gland were more extended and the alveoli, though smaller than during full lactation, were not collapsed and still had lumina,

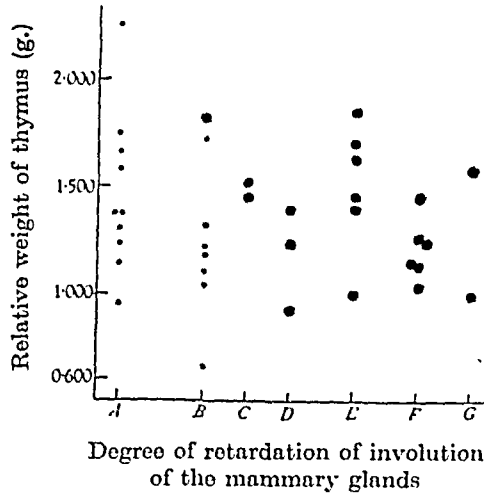


FIG. 2. Relation between relative thymus weight and degree of retardation of mammary gland involution. Small circles—control animals. Stages of mammary gland involution: *A*, complete involution with small islands of cells widely scattered in the parenchyma, collapsed alveoli with no lumina and collapsed galactophores with no milk; *B*, broader islands of cells than in *A* and galactophores that are not collapsed though containing no milk; *C*, alveoli occasionally preserved but smaller than in full lactation and the galactophores contain milk; *D*, some secretory activity evident with some alveoli containing milk and the galactophores dilated with milk; *E*, partial involution with more secretory activity and the galactophores strongly dilated with milk; *F*, a further stage of activity; and *G*, moderate involution with high secretory activity, dilated alveoli containing abundant milk and much milk in the galactophores.

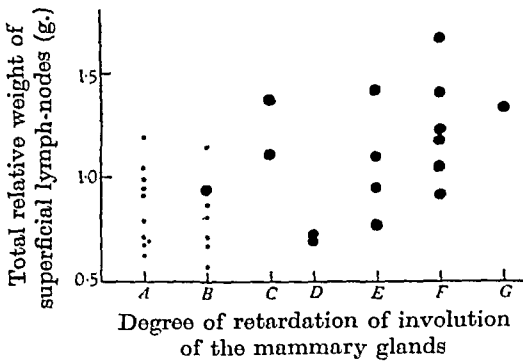


FIG. 3.

FIG. 3. Relation between relative weight of superficial lymph nodes (axillary+subscapular+inguinal) and degree of retardation of mammary gland involution (stages as in Fig. 2).

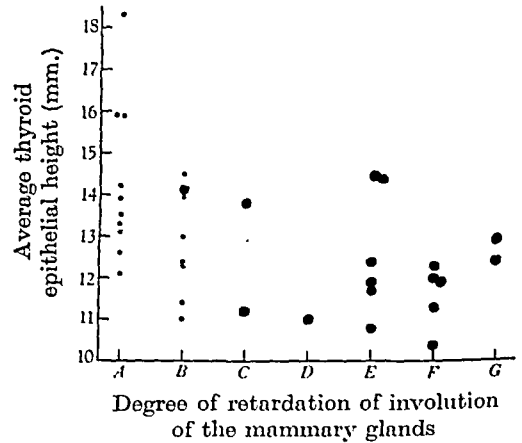


FIG. 4.

FIG. 4. Relation between average height of follicular epithelium ( $2\text{ mm.} \equiv 1\mu$ ) in the thyroid gland and degree of retardation of mammary gland involution (stages as in Fig. 2).

which were empty or contained various amounts of secretion products. Some were distended and these had flattened epithelia. Hooker & Williams [1941] found large variations in the stimulation of the mammary glands produced by prolactin. Similar

variations were found in our material, not only variations between different animals but also in the response of the different glands in the same animal or even in different parts of the same gland. In the gland showing the greatest stimulation completely involuted acini similar to those in weaned females were found in the close neighbourhood of groups of acini in full lactation. In all the rats treated with prolactin the milk ducts were strikingly distended with secretion, and colostrum corpuscles and occasional protein concretions were found.

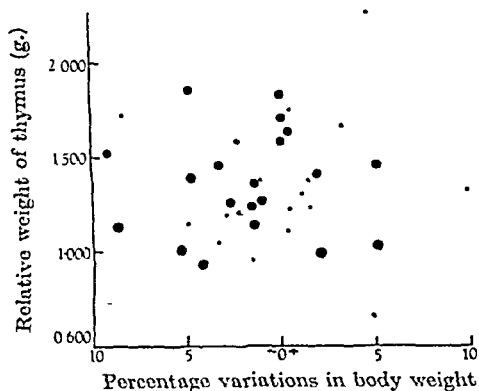


FIG. 5.

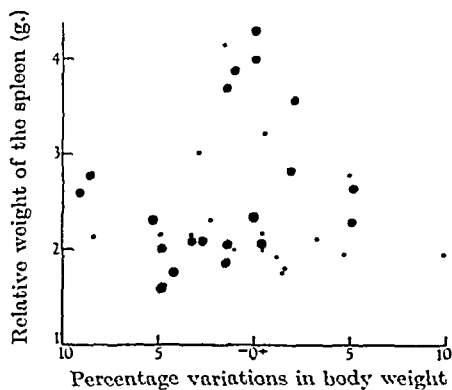


FIG. 6.

FIG. 5. Relation between relative weight of thymus and change in body weight during experiment.

FIG. 6. Relation between relative weight of spleen and change in body weight during experiment.

### Interrelations

While it is difficult to assess the secretory activity of the mammary glands, an attempt has been made to relate this to other factors by arbitrarily recognizing seven histological stages of mammary activity. Figs. 2-4 relate these stages to thymus weight, superficial lymph-node weight, and thyroid follicular epithelial height. There is evidently no relation between retarded mammary involution and thymus weight (Fig. 2), but the other two graphs (Figs. 3, 4) suggest that there may be some hypertrophy of the lymph-nodes and decrease in the epithelial height of the thyroid follicles in the animals with the least involution.

No relation could be detected between thymus or spleen weight and changes in body weight (Figs. 5, 6).

### DISCUSSION

The results show that although the prolactin injections had delayed mammary involution and in a few cases even maintained the histological picture of full lactation in rats whose litters had been removed at birth, yet the treatment did not prevent the regeneration of the thymus. The dose used has been shown by Dr Desclin (personal communication) to allow the production of deciduomata in normal females and so may be considered fully physiological.

As there was no hypertrophy of the adrenal or thyroid glands it is safe to assume that the doses used had no significant adrenocorticotrophic or thyrotrophic activity. Hooker & Williams [1941] have also reported the absence of adrenal reaction to prolactin injections.



Lactogenic hormone therefore seems not to be responsible for maintaining pregnancy involution of the thymus during normal suckling, nor can this maintenance be produced by the increase in metabolism needed to maintain partial secretory activity in the mammary glands.

The absence of adrenal hypertrophy does not rule out our previous suggestion that the maintained involution may be a result of an oversecretion of adrenocorticotrophin. (Hypertrophy of the superficial lymph-nodes may be interpreted as a local functional reaction to the increased activity and metabolism in the mammary glands of which they are tributaries—this reaction was not seen in lymphoid tissue, such as the iliac lymph-nodes, which are not dependent on the mammary glands.)

The higher degree of thyroid inactivity in the prolactin-treated spayed animals may perhaps be explained in the same way as was explained the inhibition of thyroid activity in animals suckled without milk withdrawal [Grégoire, 1946, 1947]. It may be presumed to be the expression of an altered thyrotrophin secretion under the influence of milk retention.

#### SUMMARY

1. The daily injection of 36 Riddle units of prolactin for 10 days does not prevent the regeneration of the thymus that occurs in rats which are spayed and have their litters removed at parturition.

2. The treatment had no effect on adrenal weight and seemed to enhance the decrease in thyroid activity that follows weaning.

3. It is concluded that lactogenic hormone is not the factor responsible for maintaining pregnancy involution of the thymus during suckling.

The lactogenic extract (Physolactin) was generously supplied by Mr F. A. Robinson of the Chemical Research Department, Glaxo Laboratories Ltd., Greenford, Middlesex.

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# THE INFLUENCE OF GONADAL HORMONES ON SERUM RIBOFLAVIN AND CERTAIN OTHER PROPERTIES OF BLOOD AND TISSUES IN THE DOMESTIC FOWL

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In the course of experiments on the effects of gonadal hormones on the mineral metabolism of the domestic fowl it was thought desirable to follow the changes in serum calcium and in the partition of plasma phosphorus which result from such treatment. In making these analyses it was observed that intramuscular administration of oestradiol dipropionate (Ciba) elicited a large increase in serum riboflavin [Common & Bolton, 1946]. The present paper is an account of work undertaken to confirm and extend this observation.

## METHODS

### *Objectives*

The experiments were primarily directed to securing a hormonal treatment which would simulate prematurely the reactions of the pullet to the gonadal hormones which come into play as the ovary comes naturally into full reproductive activity.

Normal development of the comb and hypertrophy of the oviduct occupy a period of some 10–14 days before the laying of the first egg. The experimental treatments were, therefore, designed to secure the appropriate degrees of hypertrophy of the oviduct and comb, and to produce the relevant changes in blood composition, within a period not greater than 14 days.

### *Experimental subjects. Birds and diet*

The White Wyandotte pullets used came from the same strain and hatching, and were reared together under the same conditions. During the experimental period the birds were housed separately in metabolism cages and received the same daily allowance (usually 100 g.) of a pelleted, all-mash, laying ration, made up in parts by weight as follows: ground wheat 25, ground yellow maize 25, ground oats 25, wheat-feed 10, dried skim milk 5, cod flakes 5, herring meal 5, cod-liver oil 1, precipitated chalk 0.75, common salt 0.25. Nutritional differences, therefore, did not exist between the different birds.

Pullets not infrequently come into lay at 16 weeks of age. This means that the ovaries may begin to come into activity from the 14th week onward. It is, therefore, desirable that pullets should not be older than 14 weeks of age at the conclusion of such experiments.

On the other hand, the use of pullets younger than 8–9 weeks of age means working with small and relatively undeveloped birds which are at a stage of very rapid growth.

For these reasons the experiments were carried out with pullets between the ages of 11 and 14 weeks.

*Hormones*

The gonadal hormones selected for use were oestradiol dipropionate (Ovocyclin P, Ciba) and testosterone propionate (Perandren, Ciba). This choice was influenced by the fact that these substances are closely related to the principal natural androgens and oestrogens and are at the same time readily available. To the best of our knowledge the actual androgens and oestrogens present in the fowl have not yet been isolated and identified.

The oestradiol dipropionate and testosterone propionate were administered in arachis oil solution by injection into the pectoralis muscles. In all cases the total volume of oil injected was brought to the same volume by addition of suitable amounts of the oil used in the hormonal preparations.\*

After consultation of the literature the levels of treatment given in Table 1 were selected. In the event the heaviest oestrogen doses used in conjunction with the androgenic treatment were found most nearly to simulate the natural hypertrophy of comb and oviduct, although at this level of treatment the changes in blood composition were exaggerated as compared with the naturally occurring phenomena.

*Analytical methods*

Serum calcium estimations were made by the modified method of Halverson & Bergeim described by Peters & Van Slyke [1932]. It is necessary to remove protein from the sera of laying or oestrogenized birds before attempting to precipitate calcium oxalate.

The fractionation of plasma phosphorus into inorganic, acid-soluble, and lipid phosphorus was performed by the technique of Youngburg & Youngburg [1930] as given by Hawk & Bergeim [1938], but the actual phosphorus determinations were made by the method of Berenblum & Chain [1938]. Serum or plasma vitellin was estimated by a method based on Laskowski's [1935] work as follows: 1 ml. plasma or serum is delivered with agitation into 9 ml. of 10 % w/v trichloroacetic acid in a 15 ml. centrifuge tube. After mixing and standing for a few minutes, the precipitate is spun down and the supernatant fluid decanted. The precipitate is then washed twice on the centrifuge with 4 % w/v trichloroacetic acid. The precipitate is then thoroughly broken up and extracted with 12 ml. of Bloor's mixture (3 vol. ethanol + 1 vol. ethyl ether), stoppered, and left overnight. Next morning the precipitate is spun down and the lipid extract decanted. The lipid extraction is repeated four times. The precipitate is then combusted with  $\text{H}_2\text{SO}_4$  conc., using  $\text{HClO}_4$  to complete the combustion, and the phosphorus determined by the method of Berenblum & Chain [1938].

Total serum carotenoids were extracted by the method of Yudkin [1941], read in a Lovibond tintometer as yellow units and converted into terms of carotene using Ferguson's graph [Ferguson, 1935].

Vitamin A was assayed in livers by the method of Davies [1933]. Riboflavin was estimated by Kodicek's [1946] fluorimetric method. In the case of serum the 'free' riboflavin was the riboflavin as assayed directly on trichloroacetic acid filtrates of the serum.

The 'crude blood volume' refers to the total amount of blood recovered by bleeding the birds freely from the external jugular. The relative cell volume was

\* The authors are indebted to Ciba Ltd., The Laboratories, Horsham, for a gift of the oil in question.

Table 1

	Pullet 33	Pullet 34	Pullet 35	Pullet 36	Pullet 37	Pullet 38	Pullet 39	Pullet 40
Dosage of <i>oestradiol</i> dipropionate (mg.)	Nil	6 x 1.0	6 x 2.0	6 x 4.0	Nil	6 x 1.0	6 x 2.0	6 x 4.0
Dosage of <i>testosterone</i> propionate (mg.)	Nil	Nil	Nil	Nil	6 x 0.75	6 x 0.75	0 x 0.75	6 x 0.75
Liver weight (kg.) at beginning of injections 14, vi, 16	1.04	1.14	1.08	1.11	1.26	1.12	1.01	1.19
Liver weight (kg.) at end of experiment 26, vi, 16	1.30	1.35	1.25	1.35	1.49	1.34	1.28	1.45
Ovary: State	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent
Weight (g.)	0.53	0.38	0.42	0.35	0.42	0.23	0.33	0.27
Oviduct: Length (cm.)	11	32	37	38	12	43	48	49
Weight (g.)	0.25	7.9	10.7	10.2	0.36	16.3	16.8	20.2
State	Undeveloped	Growing	Growing	Well-developed magnum with white corded appearance of active oviduct	Undeveloped	Well-developed magnum with white corded appearance of active oviduct	Well-developed magnum with white corded appearance of active oviduct	Apparently fully developed and like fully active oviduct
Cruel blood volume:								
(ml.)	32	47	50	80	34	44	55	86
(ml./kg. live weight)	25	35	40	59	23	33	43	59
Haematocrit value	28.9 (26.5)*	31.1 (24.9)	22.7 (23.1)	19.1 (27.4)	30.4 (21.6)	32.6 (24.8)	28.9 (21.6)	21.9 (23.0)
Total serum lipid (g./100 ml.)	0.64	1.01	8.78	11.34	0.65	0.78	3.61	11.08
Serum Ca (mg./100 ml.)	12.7 (12.3)	23.4 (12.2)	61.4 (13.0)	93.5 (12.6)	13.5 (12.6)	22.8 (12.9)	41.8 (12.8)	95.5 (12.8)
Plasma total acid-soluble P (mg./100 ml.)	6.8 (5.4)	8.5 (5.1)	16.9 (5.6)	16.8 (5.0)	7.3 (5.4)	5.8 (5.6)	11.2 (5.7)	14.3 (6.3)
Plasma inorganic P (mg./100 ml.)	6.3 (5.1)	7.4 (5.1)	15.7 (5.8)	15.7 (4.6)	6.3 (5.1)	5.0 (5.1)	10.3 (5.3)	13.1 (5.1)
Plasma vitellin P (mg./100 ml.)	<0.1 (-0.4)	5.3 (0.2)	23.0 (-0.6)	37.5 (-0.2)	<0.1 (0.5)	3.0 (0.6)	12.5 (-0.6)	46.0 (-0.5)
Plasma phospholipid P (mg./100 ml.)	7.1 (7.1)	15.1 (6.0)	86.8 (7.0)	130.8 (6.7)	7.2 (5.1)	11.9 (5.8)	41.7 (6.2)	114.4 (6.7)
Serum carotenoids, as 'carotene' (mg./100 ml.)	0.24	0.42	0.84	0.92	0.28	0.34	0.50	0.82
Serum riboflavin:								
Total (µg./ml.)	0.03	0.62	2.65	5.32	0.17	0.07	1.45	4.94
Acid-soluble (µg./ml.)	0.07	0.16	2.05	4.00	0.06	0.04	1.50	4.73
Liver riboflavin (µg./g.)	24.4	29.0	24.6	23.3	28.2	32.0	25.6	22.9
Liver fatty acids (g./100 g.)	2.0	3.4	4.0	4.4	2.1	3.0	3.2	3.8
Liver vitamin A (Lloyd's n.e.g.)	18.7	14.5	23.2	22.9	19.3	17.5	17.8	15.8

\* The figures in brackets relate to analyses made immediately before beginning the hormonal treatments. Plasma vitellin was not determined directly in the case of these initial samples, and the figures quoted in brackets for this constituent are the values for total P less acid-soluble P and lipid P. The resultant estimates of plasma vitellin are subject to the cumulative errors of the other analytical figures, but they are sufficient to confirm that little or no vitellin was present in the plasma of any of the pullets at this stage. The other values for plasma vitellin P are direct estimates.

estimated by centrifugation of oxalated blood, care being taken to add the same amounts of oxalate in all cases; the results obtained are referred to below as haematocrit values, although a haematocrit tube was not used in the evaluation.

## RESULTS

### *Experiment 1*

In this experiment the doses were divided into six equal injections administered on alternate days, beginning when the birds were 82 days old. The effect of the androgenic treatments on the combs was perceptible 30 hr. after the first injection. The pullets were killed before the morning feed on the second day after the last dose, when they were 94 days old.

#### *Comb and wattle development*

The combs and wattles of pullets 33-36, which did not receive any androgen, remained immature, except that there was a distinct though slight tendency to regression with increasing oestrogen dosage. The combs and wattles of pullets 37-40, which all received the same dosage of androgen, hypertrophied to the greatest extent in the case of pullet 37 (receiving no oestradiol dipropionate) and to a slightly lesser extent in the birds receiving oestrogen. In pullet 40 the appearance at the conclusion of the experiment closely resembled that of a pullet at about the time of laying her first egg.

The observations on comb and wattle development demonstrated that in this respect, and at the dosages used, oestradiol dipropionate slightly antagonized the action of testosterone propionate.

#### *Ovaries*

Accurate dissection of the ovaries was not attempted, but the appearance and weights confirmed that they were in all cases in a quiescent state. So far as they go, the observations accord with the known restrictive effects of oestrogen on ovarian growth.

#### *Oviducts*

In pullets 33 and 37 (no oestradiol dipropionate) the oviducts were completely undeveloped. Oestrogen treatment induced hypertrophy. It would seem from a comparison of pullets 33-36 with pullets 37-40 that androgen augmented the effects of oestrogen in regard to hypertrophy of the oviduct. (This observation confirms similar observations made in the course of other experiments in this laboratory.) Witschi & Fugo [1940] have described an oviduct-stimulating effect of androsterone or testosterone in the starling, and Bloom, McLean & Bloom [1942] have described a synergism of testosterone propionate and oestradiol in inducing medullary bone formation in castrate male pigeons. These observations support the conception of androgen and oestrogen being normally concerned in the development of the pullet's oviduct. In the case of pullet 40 the oviduct closely resembled the fully active oviduct and this pullet most closely simulated the natural reproductive state in this respect as well as in comb and wattle development.

*Crude blood volume*

The data presented in Table 1 show that the volume of blood secured by direct bleeding was greatly increased by oestrogen treatment. The magnitude of the effect strongly suggests that an actual increase of the volume of the circulating blood was involved. It is known that all three types of gonadal hormones and adrenal cortical hormones influence the excretion of water, sodium, and chlorine [Burrows, 1945], and the present observations conform with these facts in so far as blood volume appears to have been increased. It is, of course, possible that this increase was a consequence of the action of the injected oestradiol dipropionate on the pituitary, leading to increased secretion of adrenotrophic hormone with consequent increased adrenal activity.

There is no doubt that the haematocrit value was reduced by the heavier oestrogen treatments, that is, those which most nearly simulated the natural reproductive state in respect of comb growth and hypertrophy of the oviduct. While it is believed that oestrogen checks formation of red blood cells [Burrows, 1945], counts or haematocrit readings are of themselves evidently insufficient to demonstrate this point in view of the changes which may take place in blood volume. In the present experiments it would appear that red cell formation was insufficient to maintain the normal non-reproductive haematocrit value in the more heavily oestrogenized pullets. Less certain are the indications that the lowest dose of oestrogen produced a slight increase in haematocrit value. In view of the well-known reversal of effects which may obtain with different levels of gonadal hormones, this may well be a genuine effect.

*Serum calcium and plasma phosphorus*

The data for these are presented principally because they assist in defining the state of the pullets.

The very high levels of serum calcium in pullets 36 and 40 are similar to those reported by other workers for oestrogenized fowls [Zondek, 1939; Zondek & Marx, 1939]. They are, however, higher than those normally encountered during the bird's reproductive cycle, and to that extent the simulation of the normal pre-laying condition was imperfect. In the normal cycle, although comb growth and hypertrophy of the oviducts are, if anything, greater than in pullets 36 and 40, nevertheless serum calcium is not increased to the same extent.

The picture presented by the phosphorus partition confirms that observed by McDonald & Riddle [1945] in oestrogenized pigeons. The values attained in pullets 36 and 40 are, however, higher than the average figures recorded for laying hens [Laskowski, 1935; Roepke & Hughes, 1935], and are similar to those obtained by Laskowski [1938] for the plasma of non-laying hens injected with anterior pituitary gonadotrophin, though somewhat higher. The large increases in plasma inorganic phosphorus brought about by the higher doses of oestrogen agree with similar observations made on the fowl by Avery, Scott & Conrad [1940]. It may be surmised that this increase is mainly in the non-ultrafiltrable form, as has been shown to be the case in the oestrogenized pigeon [McDonald & Riddle, 1945].

The response of plasma phosphorus fractions thus presents the same imbalance relative to comb development and hypertrophy of the oviduct as does the response of serum calcium. These facts might be interpreted as meaning either that the

treatments did not closely reproduce the intensity of the natural oestrogenic plus androgenic influences at work in the normal cycle or that in the normal cycle an additional influence is at work which to some extent offsets the effect of oestrogen in raising serum calcium. Fleischmann & Fried [1945] have found that when thyroxine (1 mg. daily) is injected into immature chicks simultaneously with oestradiol propionate (1 mg. daily) then increases in serum calcium or in inorganic lipid, or vitellin phosphorus do not take place; growth of the oviduct, however, is not similarly inhibited by thyroxine administered simultaneously with oestrogen. Similar observations on pigeons have been reported by McDonald, Riddle & Smith [1945]. It would seem reasonable, therefore, to interpret the results of the gonadal hormone treatments used in the present experiment as being a fairly close simulation of the normal so far as secretion of gonadal hormones by the active ovary is concerned, but with the reservation that in the normal case additional factors as well as gonadal hormones come into play, one at least of which probably is an increased thyroid activity limiting the changes in blood chemistry induced by gonadal hormones.

#### *Serum carotenoids*

The data are sufficient to demonstrate an increase under the influence of gonadal hormones which may be associated with the concurrent mobilization of fat and phospholipid.

#### *Serum and liver riboflavin*

Precautions were taken against losses by photolysis before making these determinations. This may explain the fact that higher levels of serum riboflavin were observed for given treatments than in the preliminary observations already reported [Common & Bolton, 1946]. The increases in serum riboflavin brought about by hormonal treatment were of a very large order. The data for the livers suggested that the more intense hormonal treatments brought about some reduction in the concentration of riboflavin in the liver. Rough estimations of the total liver fatty acids showed a lipotropic effect of the hormonal treatments. Unfortunately, the weights of the livers were not recorded, so that the total riboflavin contents of the livers can only be estimated roughly by assuming liver weights of 30–40 g. However, calculations made on this assumption are sufficient to show that, in the most heavily oestrogenized pullets, the total amounts of serum riboflavin were about 40 % of the total amounts in the livers. Taken in conjunction with the order of magnitude of the changes in liver riboflavin concentration, these circumstances suggest that the high levels of serum riboflavin in the oestrogenized pullets are not readily to be explained by displacement of riboflavin from the livers consequent upon lipid mobilization.

Comparison of the data for total and 'free' riboflavin in the serum suggests that by far the greater part if not all the serum riboflavin is present as 'free' riboflavin, or as compounds of riboflavin which are readily hydrolyzed in the cold by 10 % w/v trichloroacetic acid.

It may be observed that estimations of total serum aneurin by the thiochrome method of Harris & Wang [1941] did not reveal any comparable effects of the hormonal treatments in mobilizing this substance. If any such mobilization of aneurin occurs, therefore, it must be to a relatively lesser degree than in the case of riboflavin.

*Experiment 2*

The effects observed in the preliminary experiment [Common & Bolton, 1946] were of such a striking nature that a supplementary experiment was conducted using two control pullets (nos. 41 and 42) and two pullets receiving a level of oestradiol dipropionate plus testosterone propionate comparable with that administered to pullet no. 40 in Exp. 1.

The experiment was conducted exactly as in Exp. 1, except that the total dosage of hormones was administered in thirteen equal daily doses. The birds came from the same hatching as those used in Exp. 1, but they were 98 days old when killed at the end of the experiment. Greater attention was paid to the liver analyses. The results are summarized in Table 2.

Table 2

	Pullet 41	Pullet 42	Pullet 43	Pullet 44
Dosage of oestradiol dipropionate (mg.)	Nil	Nil	13 × 2.5 mg.	13 × 2.5 mg.
Dosage of testosterone propionate (mg.)	Nil	Nil	13 × 0.5 mg.	13 × 0.5 mg.
Live weight (kg.) at beginning of injections, 16. vi. 46	0.92	0.82	0.79	0.92
Live weight (kg.) at end of experiment, 30. vi. 46	1.12	0.99	0.98	1.07
Ovary: State	Quiescent	Quiescent	Quiescent	Quiescent
Weight (g.)	0.34	0.21	0.20	0.29
Oviduct: Length (cm.)	10	10.5	51	56
Weight (g.)	0.20	0.22	16.4	16.1
State	Undeveloped	Undeveloped	Fully functional appearance; magnum very soft	Fully functional appearance; magnum very soft
Crude blood volume (ml.)	31	31	53	57
Total serum lipid (g./100 ml.)	0.59	0.55	13.88	13.94
Serum Ca (mg./100 ml.)	12.7	12.9	87	102
Serum vitellin P (mg./100 ml.)	0.1	0.1	54.1	68.5
Serum carotenoids, as mg. 'carotene' / 100 ml.	0.36	0.38	0.92	0.90
Serum riboflavin:				
Total (μg./ml.)	0.10	0.06	3.61	3.87
'Acid-soluble' (μg./ml.)	0.01	0.01	3.54	3.72
Liver: Weight (g.)	26.1	21.2	31.1	31.9
Appearance	Normal	Normal	Pale	Pale
Liver dry matter (%)	26.92	26.32	27.38	27.44
Total liver fatty acids (%)	2.73	2.37	3.27	4.54
Total liver fatty acids (% of dry matter)	10.33	9.01	11.95	16.53
Liver riboflavin (total μg./g.)	28.0	30.7	23.2	22.9
Spleen: Weight (g.)	1.91	1.82	1.23	0.97
Appearance	Red, normal	Red, normal	Pale and pinkish, smaller than normal	Pale and pinkish, smaller than normal

The observations on comb growth, etc., were in general consistent with those of Exp. 1, especially in so far as they relate to pullets 33 and 40. The oviducts of these two birds were fully developed, and it was noticed that the cloacae were moist and appeared swollen and relaxed as compared with those of nos. 41 and 42. The blood



analyses also conformed with previous results, but were extended to include an estimation of total serum lipid with the results cited in Table 2. Lorenz, Chaikoff & Entenman [1938] have recorded similar large increases in the total lipids of whole blood of oestrogenized hens, values in excess of 1000 mg./100 ml. being frequently found. The serum of pullets under strong oestrogenic influences evidently constitutes a remarkable colloidal system, for its total lipid content may, as in the present experiment, approach 14 g./100 ml. The results for serum riboflavin agreed with those secured with pullets 33 and 40, except that the levels attained in pullets 43 and 44 were not quite so high as in pullet 40. The data for the concentration of riboflavin in the livers suggest that the hormonal treatment led to a decrease in concentration. Such a decrease might at first sight be taken as indicating that the increased serum riboflavin of the oestrogenized pullet is a consequence of mobilization of liver riboflavin. Consideration of the actual amounts of riboflavin involved, however, suggests that this view is open to question. It is possible to secure a measure of the total amounts of riboflavin involved because the volumes of the sera were known to within about 1 ml. The relevant figures are set out in Table 3; they demonstrate that the

Table 3. *Riboflavin contents of livers and sera secured by free bleeding*

	Pullet no. ...	41	42	43	44
Volume of serum (ml.)		22	22	42	46
Serum riboflavin ( $\mu\text{g./ml.}$ )		0.10	0.06	3.61	3.87
Total serum riboflavin ( $\mu\text{g.}$ )		2.2	1.3	152	178
Weight of liver (g.)		26.1	21.2	31.1	31.9
Liver riboflavin ( $\mu\text{g./g.}$ )		28.0	30.7	23.2	22.9
Total liver riboflavin ( $\mu\text{g.}$ )		731	651	722	731

sera of the untreated pullets contained amounts of riboflavin of the order of 0.3 % of the amounts in the livers, whereas the sera of the treated pullets contained amounts of the order of 20 % the amounts in the liver.

Now it will be seen that if the amounts of riboflavin in the sera of nos. 43 and 44 were added to those in the livers, then the liver concentrations would be raised from 23.2 to 28.1  $\mu\text{g./g.}$  in the case of no. 43 and from 22.9 to 28.4  $\mu\text{g./g.}$  in the case of no. 44. These calculated values are of the same order as those found in the untreated pullets. We do not, however, regard this as necessarily indicating depletion of liver reserves, and this for the following reasons. It is not improbable that the greater weights of the livers of nos. 43 and 44 as compared with nos. 41 and 42 are due to the effects of oestrogen; Gericke [1945-46] has adduced evidence that the livers of high-producing hens are considerably heavier than those of low-producing hens; the former presumably have the more active ovaries and are, therefore, subject to more intensive oestrogenic influence. Thus the lower liver concentration of riboflavin in nos. 43 and 44 might be an expression of an increase in liver weight without a corresponding increase in riboflavin content.

Again, the total riboflavin content of normal eggs analysed in this laboratory has been of the order of 200  $\mu\text{g.}$  Hence if the effects of oestrogen are interpreted as indicating the operation of a mechanism whereby liver riboflavin reserves are mobilized against demands for egg production, then it is evident that such a mechanism could meet those demands only to a limited extent, for the total liver riboflavin is adequate for production of but a few normal eggs, nor does the available

evidence show that the liver can accumulate any considerable reserve of riboflavin [Bolton, 1944].

It seems to us more feasible, therefore, to regard the increased serum riboflavin as corresponding to changes in the metabolism and transport of riboflavin whereby the blood becomes capable of maintaining a higher concentration against the relatively heavy demands for egg formation, and that these changes are independent of any concurrent mobilization of the limited reserves of the liver. These considerations incline us to the view that the possibility of extra-hepatic sources of increased serum riboflavin of pullets subject to strong oestrogenic influences requires serious consideration. One possible source would be an increased capacity for absorption and retention of riboflavin from the alimentary tract.

In conclusion it may be remarked that the spleens of the treated birds evinced differences in weight and macroscopic appearance as compared with those of the two controls.

#### SUMMARY

1. An experiment is described in which graded doses of oestradiol dipropionate with a superimposed dose of testosterone propionate were employed to simulate in the immature pullet the comb development, hypertrophy of the oviduct, and changes in blood composition which attend the normal development of reproductive activity.

2. Treatments with oestradiol dipropionate plus testosterone propionate which simulated normal reproductive hypertrophy of the pullet's oviduct, comb, and wattles also elicited the concomitant increases in serum calcium and various plasma phosphorus fractions. The blood changes, however, were exaggerated in comparison with those occurring as the pullet enters normal reproductive activity. It is suggested that a closer simulation of the normal changes would have required supplementation with another hormonal treatment, most probably thyroid in nature.

3. It is shown that to the known effects of such treatment the following effects may be added:

- (a) a decrease in haematocrit value;
- (b) an increase in the yield of blood and probably also in total blood volume;
- (c) a very large increase in serum riboflavin;
- (d) a decrease in the size of the spleen and an alteration in its macroscopic appearance.

4. The form in which this serum riboflavin occurs and the question of the immediate source from which it is derived are discussed.

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## ADDENDUM

(11 December 1946)

Since the completion of the work described above, we have been able to make preliminary observations on the serum riboflavin of hens from a flock maintained on grass runs under normal semi-intensive conditions of management. Laying birds were identified by their laying records. Non-laying hens were discriminated from those coming into lay by appearance and handling, confirmed by subsequent records. The data may be summarized as follows:

Class	No. of birds	Total serum riboflavin ( $\mu\text{g./ml.}$ )	
		Average	Range
Cocks (adult)	3	0.07	0.06-0.08
Non-laying hens	6	0.06	0.05-0.10
Hens coming into lay	6	0.84	0.17-2.54
Laying hens	5	0.86	0.55-1.18

These data strongly suggest that serum riboflavin varies in the hen according to the stage of the reproductive cycle, being relatively high in laying birds and in those approaching laying, and relatively low in sexually inactive hens. The values for sexually inactive hens appear to be comparable with those obtained for cocks.

It may reasonably be inferred that these variations in serum riboflavin of hens at different stages of reproductive activity and kept under normal commercial conditions are controlled by endogenous gonadal hormone activity.

# EFFECT OF 2-THIOURACIL ON THE HISTOLOGY OF THE NORMAL HUMAN THYROID

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Descriptions of the effect of thiouracil or thiourea upon the histology of the human thyroid have until now been limited to cases of Graves's disease [Moore, Sweeney, Cope, Rawson & Means, 1944; Shirer & Cohen, 1945; Halpert, Cavanaugh & Keltz, 1946]. In the course of a study of thiouracil therapy in heart failure the opportunity was taken to study in humans the histological stimulation first observed by MacKenzie & MacKenzie [1943] and Astwood, Sullivan, Bissell & Tyslowitz [1943] in the experimental animal, to find the degree of thyroid hyperplasia and colloid depletion evoked by increasing dosage with thiouracil, and to see at what stage of treatment changes are recognizable and how effectively the thyroid reverts to normal when the drug is stopped. This paper describes the changes in normal human thyroids and in one case the effect of additional thyrotrophic extract was studied. By 'normal', no more is implied than the absence of clinical signs of goitre or Graves's disease.

## MATERIAL

The material was taken from a series of ten ultimately fatal cases of heart failure of varying aetiology treated with thiouracil. Data on basal and resting oxygen consumption, cardiac output and other circulatory measurements have been published elsewhere [Sharpey-Schafer, 1946].

The patients are divided into three groups: (I) those who received a total dose greater than 70 g.; (II) those who received less than 70 g.; and (III) those who died some months after the drug was stopped. In view of the post-mortem nature of most of the material, a histological rather than a cytological study was made and a qualitative rather than a statistical analysis. The terms small, medium-sized and large follicles refer to those whose mean diameter is about 150, 300 and 500  $\mu$  or more. Low cuboidal, cuboidal, and high cuboidal refer to cells of about 6, 15 and 20  $\mu$  or more in height.

## FINDINGS

The data are summarized in Table 1.

Group I showed a striking and more uniform hyperplasia of the thyroid than the other groups (Pls. 1, 2, figs. 1-6). The follicles were more closely packed and their lining cells taller. The average follicular lining cell was both broad and tall and best described as high cuboidal in type. Papilliform infoldings were present in case 1 (Pl. 1, fig. 1) and to a slight extent in case 2, but not in cases 3 and 4. Columnar cells were seen in case 1 (Pl. 1, fig. 2) and occasionally in cases 2 and 3. The greatest colloid depletion was seen in case 1 and more mitoses were seen in cases 1 and 2

Table 1

## Thyroid histology

Case no.	Sex and age	Disease	Thiouracil dosage	Time interval between last dose of thiouracil and death	Weight of thyroid in g.	Thyroid histology							Desqua- mation of follicular epithelium	Inter- stitial fibrosis	Inter- stitial lymphoid tissue
						Variation in follicle size	Average follicle size	Average follicle cell height	Colloid storage	Mitoses					
Group I: patients who received more than 70 g. of thiouracil															
1*	F. 45	Mitral stenosis	76 g. in 38 days	Nil	Surgical biopsy	Slight	Small	Tall	Almost nil	Occasional	Nil	Nil	Nil	Nil	
2	F. 66	Hypertension	84 g. in 48 days	Nil	30	Slight	Small	Tall	Slight	A fair number	Marked	Nil	Nil	Nil	
3	M. 52	Hypertension	308 g. in 154 days	Nil	19	Slight	Small	Tall	Slight	Occasional	Marked	Nil	Nil	Nil	
4	M. 56	Cor pulmonale	337 g. in 180 days	Nil	Surgical biopsy	Slight	Small	Tall	Slight	Occasional	Marked	Nil	Nil	Nil	
4	M. 56	Cor pulmonale	349 g. in 192 days (+ 12 ml. of thyrotrophin)	Nil	35	Slight	Small	Tall	Slight	Occasional	Very marked	Nil	Nil	Nil	
Group II: patients who received less than 70 g. of thiouracil															
5	M. 59	Aortic in-competence	55 g. in 55 days	Nil	—	Well marked	Small	Medium	Slight	Nil	Moderate	Nil	Nil	Nil	
6	F. 45	Cor pulmonale	44 g. in 34 days	Nil	28	Well marked	Medium	Medium	Moderate	Nil	Moderate	Nil	Nil	Nil	
7	F. 63	Hypertension and empyaema	38.4 g. in 42 days	4 days	40	Well marked	Medium	Low	Marked	Nil	Nil	Slight	Nil	Nil	
8	F. 39	Mitral stenosis	18 g. in 18 days	Nil	10	Well marked	Medium	Medium	Slight	Nil	Moderate	Nil	Nil	Nil	
9	F. 34	Mitral stenosis	8 g. in 4 days	Nil	30	Medium	Large	Low	Marked	Nil	Nil	Nil	Nil	Nil	
Group III: patients who died many weeks after the thiouracil course was finished															
1(a)*	F. 45	Mitral stenosis	336 g.	170 days	30	Well marked	Medium†	Low	Marked	Nil	Nil	Marked	Nil	Nil	
10	F. 55	Cor pulmonale	122 g.	169 days	20	Well marked	Large	Low	Marked	Nil	Nil	Moderate	Nil	Nil	

\* Case 1 in Group I and 1(a) in Group III represent respectively a surgical biopsy specimen taken during thiouracil therapy and a post-mortem specimen 170 days after the drug was stopped, both from the same patient.

† 1 hyperplastic islet seen.

(Pl. 1, fig. 4) than in cases 3 and 4 (Pl. 2, fig. 6). Whether the colloid present is residual or newly formed one cannot say. But in view of the greater colloid depletion in case 1 (after 76 g. of thiouracil) and the fewer mitoses and increased desquamation in cases 3 and 4 (after 308 and 349 g. of thiouracil), the possibility might be considered that the colloid-depleting and cell-stimulating effect of thiouracil therapy is best seen histologically as the drug effect is rising to a maximum and that it then tails off.

The follicles in group II were more loosely knit and much less uniform than in group I. Though the colloid depletion and reduction in average follicle size in cases 5, 6 (Pl. 2, figs. 7, 8) and 8 might be considered evidence of stimulation, a similar picture has been seen by us in post-mortem examinations of cases not treated by thiouracil. Without knowledge of the pre-treatment histology of these glands it was not possible, therefore, to attribute hyperplastic changes to the thiouracil: the normal variability of the histology of the thyroid had also to be discounted. This is in direct contrast to the group I picture which was *not* seen in control cases. The early changes in the thyroid histology are likely to be dependent upon its previous state, especially its degree of colloid storage, and it is probable that the thyroid of case 8 may have been in an active phase before treatment was started, whereas case 7, whose thyroid showed no evidence of stimulation and weighed 40 g., had a colloid goitre. Thus there is only clear histological evidence of 'thiouracil stimulation' in those cases receiving more than 70 g. of the drug. It is of interest to note that in spite of the well-known goitrogenic property of thiouracil the average weight of the thyroid glands in group I was 28 g. (the largest weighed 35 g.), in group II 27 g. and in control post-mortem material 23 g. (average of 100 cases). In this small series of human cases, therefore, thiouracil did not always prove goitrogenic.

The findings in group III prove that colloid repletion takes place when thiouracil is stopped and is apparently associated with an interstitial fibrosis. The residual islet of activity in case 1 suggests that recovery is a slow process.

The desquamation of the follicular epithelium, probably exaggerated by post-mortem change, which appeared more marked with increasing doses of thiouracil, was also noted by Astwood *et al.* [1943] in the thyroids of some of his thiourea-treated rats. It might be an 'over-stimulation' or exhaustion phenomenon.

#### DISCUSSION

It is now generally accepted that thiouracil interferes with the formation of thyroxine and that the resultant low blood-thyroxine level leads to the production of increased thyrotrophic hormone by the anterior pituitary [Astwood *et al.* 1943; MacKenzie & MacKenzie, 1943]. Thus, as expected, the thyroid hyperplasia following thiouracil treatment resembles that produced by direct administration of thyrotrophic hormone. This is illustrated in Pl. 3, fig. 11, which shows a stimulated human thyroid from a patient, aged 58, with carcinomatosis, given intramuscular injections of thyrotrophin (7 ml. of Ambinon\* in 3 days). The reaction was much quicker and contrasts with the negligible changes seen in case 9 (8 g. of thiouracil in 4 days). It is understandable that the 'thiouracil' mechanism of indirect production of thyrotrophic hormone would demand a fair latent period to produce gross histological changes.

\* The thyrotrophin used throughout was Ambinon (Organon) containing 200 Heyl-Laqueur units per ml.

The appearance of the thyroid in case 4, before and after thyrotrophic extract given on top of a massive course of thiouracil (over 300 g.), showed no obvious extra stimulation. If anything, it showed extra desquamation. Here, at the other end of the scale, is suggestive evidence that the patient's effective thyrotrophic hormone production had already reached its maximum.

An interesting finding was the absence of interstitial lymph follicles and collections of lymphocytes in the stimulated thyroids, in contrast to their presence in Graves's disease both untreated and after thiouracil [Moore *et al.* 1944]. On the other hand, sections of thyroid from six cases of Graves's disease not treated by iodine were examined and all found to contain numerous areas of hyperplasia of a similar type to that seen in the group I cases. However, the 'thyrotoxic' thyroids showed a larger average follicle size, more columnar-celled follicular epithelium and more papilliform infoldings than the 'thiouracil' thyroids.

#### SUMMARY

Two surgical biopsies and ten post-mortem specimens of thyroid gland were examined histologically from non-thyrotoxic patients with heart failure, who had received total doses varying from 8 to 349 g. of 2-thiouracil. One case received additional thyrotrophic extract.

Clear-cut evidence of stimulation (closely packed, small, colloid-depleted follicles lined by a high cuboidal epithelium) was seen only in the four cases who received more than 70 g. This hyperplasia was similar to that seen in Graves's disease and following thyrotrophic extract administration, but appeared much more slowly than with the latter. The hyperplastic glands showed no interstitial lymphoid collections. Their average weight (28 g.) was not significantly greater than that of untreated control cases (23 g.).

Within a few months of cessation of thiouracil therapy (two cases) colloid reformed, the follicular epithelium was reduced in height and there was an interstitial fibrosis. One hyperplastic islet was still to be seen (one case).

It appears possible that 'thiouracil stimulation' reached a maximum (the dosage lying somewhere between 70 and 300 g.), after which no further hyperplasia could be elicited either by continuing the drug or, in one case, by administering thyrotrophic extract. Desquamation of the follicular epithelium into the follicle lumina was a marked feature of the most-stimulated thyroids (three cases).

We are indebted to Prof. J. H. Dible for the loan of the slides from six cases of Graves's disease not treated with iodine; to Mr E. V. Willmott, A.R.P.S., for the photomicrographs; and to Mr J. R. Baker, A.I.M.L.T., and Mr J. G. Griffin, A.I.M.L.T., for the histological preparations.

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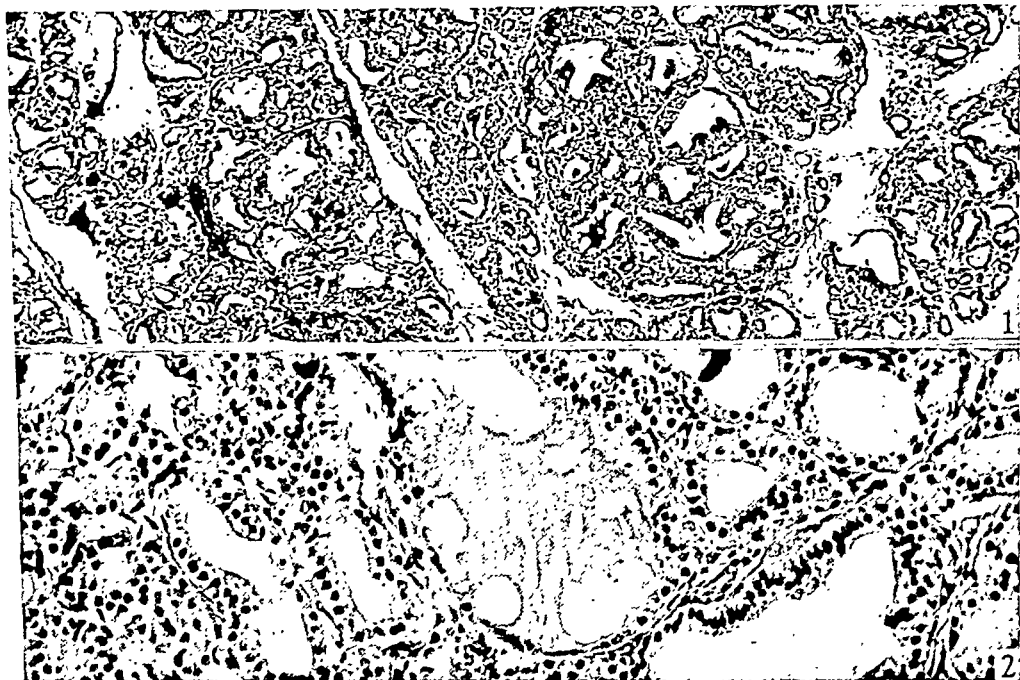


FIG. 1 above  $\times 45$ .

Case 1

FIG. 2 below  $\times 190$ .

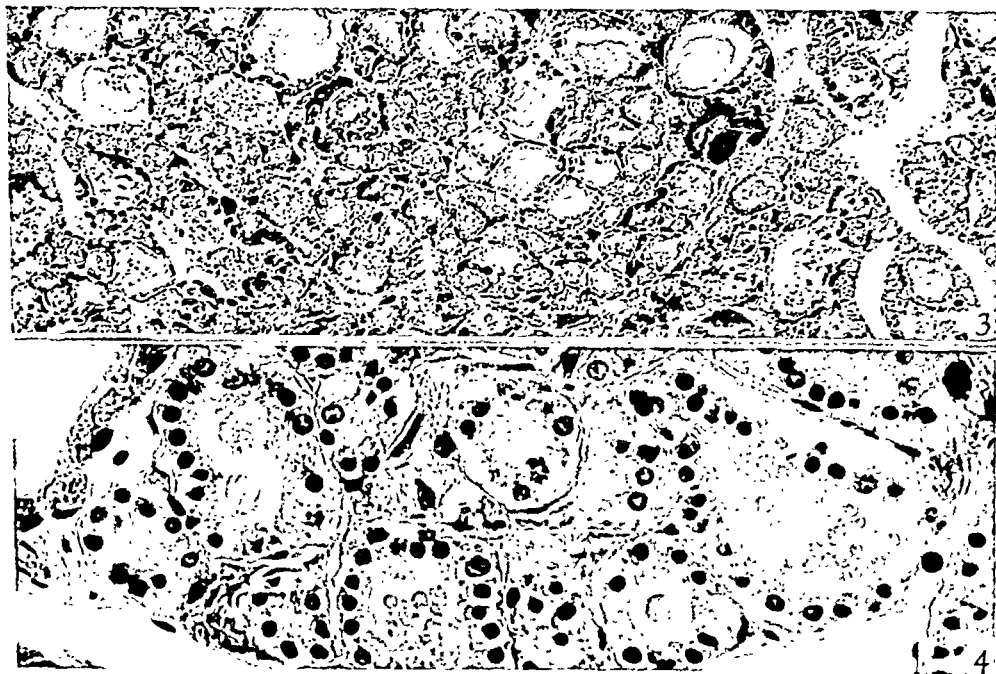


FIG. 3 above  $\times 45$ .

Case 2

FIG. 4 below  $\times 400$ .



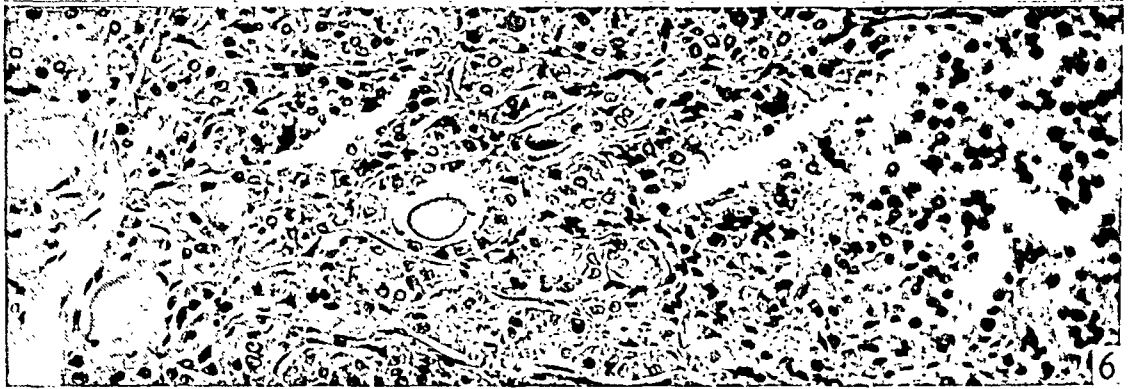
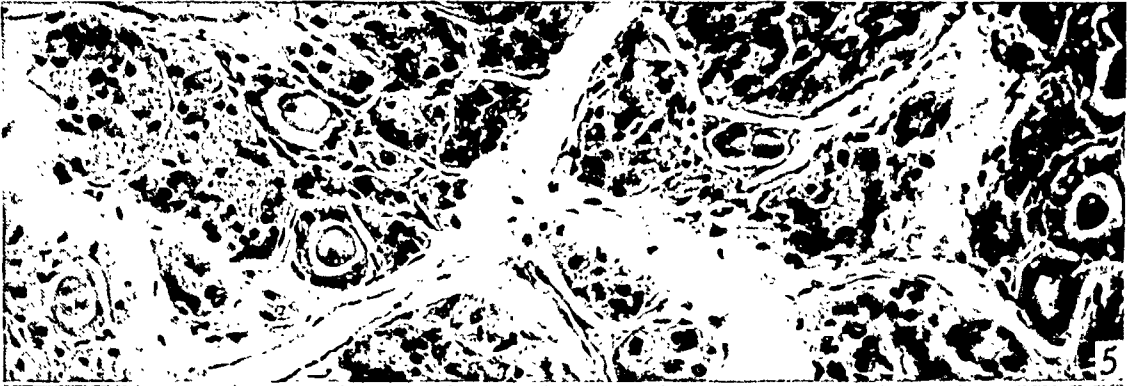


FIG. 5 above  $\times 180$ .

CASE 4

FIG. 6 below  $\times 180$ .

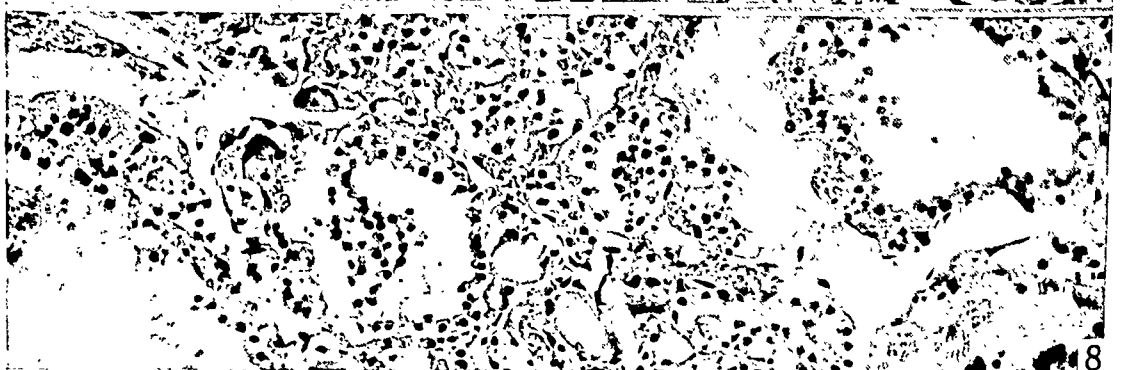
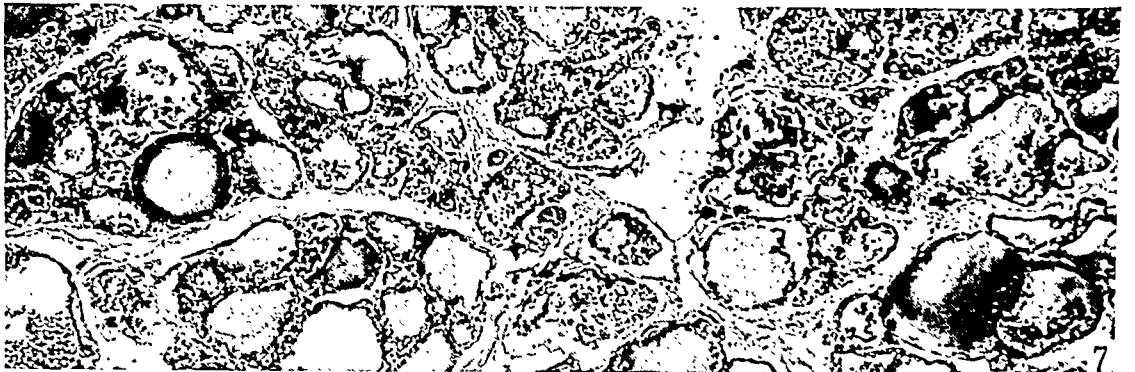


FIG. 7 above  $\times 45$ .

CASE 6

FIG. 8 below  $\times 190$ .

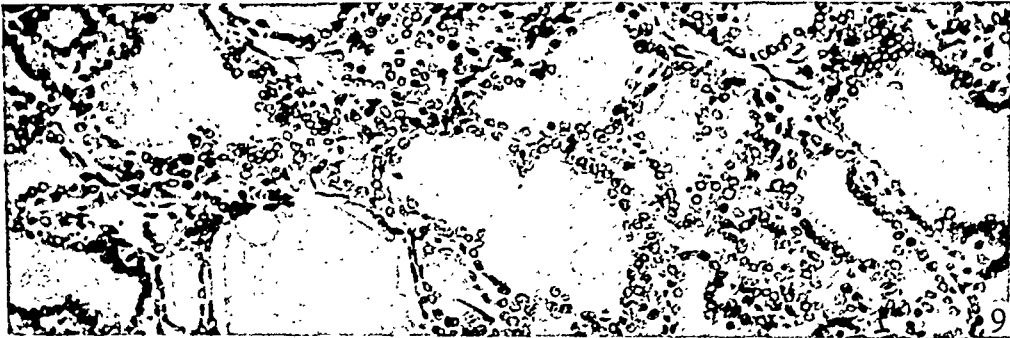


FIG. 9 above  $\times 190$ .

Case 1 (a)

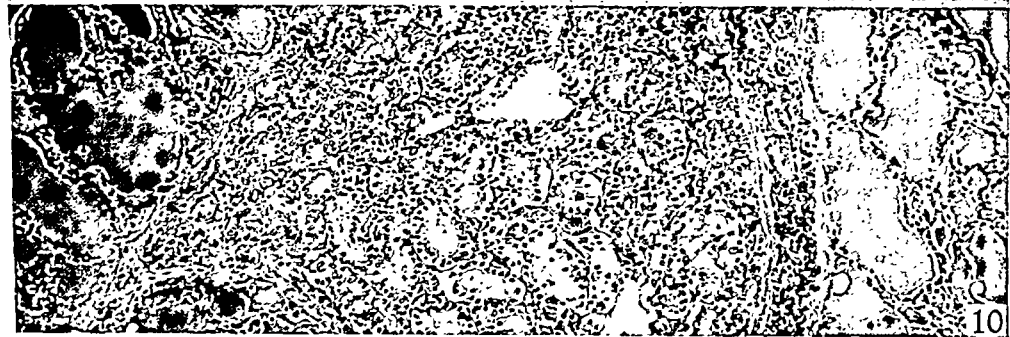


FIG. 10 below  $\times 90$ .

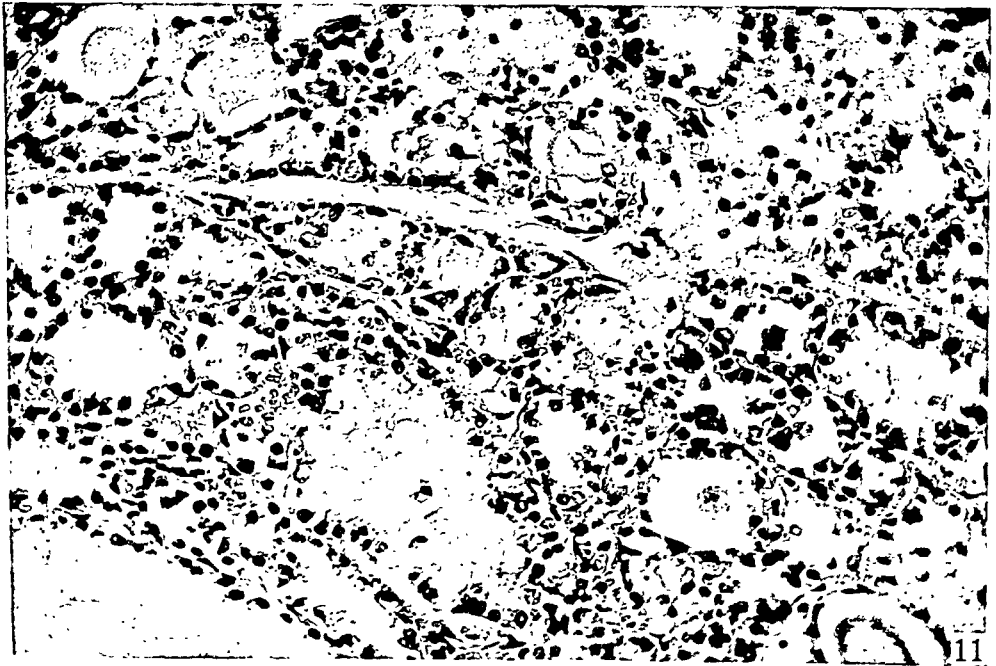


FIG. 11  $\times 275$ .



## EXPLANATION OF PLATES

Photomicrographs of thyroid glands all stained by haematoxylin and eosin.

## PLATE 1

FIG. 1.  $\times 45$ , case 1. Surgical biopsy after 76 g. of thiouracil, formol-saline fixation. Small and medium-sized follicles. Fairly uniformly distributed and mostly depleted of colloid.

FIG. 2.  $\times 190$ , case 1. Surgical biopsy after 76 g. of thiouracil, formol-saline fixation. High-cuboidal and columnar follicular epithelium, the central colloid-containing follicle showing a mixture of high and flattened lining cells.

FIG. 3.  $\times 45$ , case 2. Specimen 24 hr. *post mortem* after 84 g. of thiouracil, formol-Zenker fixation. Small and medium-sized follicles. Fairly uniformly distributed and mostly depleted of colloid.

FIG. 4.  $\times 400$ , case 2. Specimen 24 hr. *post mortem* after 84 g. of thiouracil, formol-Zenker fixation. High-cuboidal follicular epithelium, the central follicle showing a mitotic figure.

## PLATE 2

FIG. 5.  $\times 180$ , case 4. Surgical biopsy after 337 g. of thiouracil, formol-Zenker fixation. Small follicles, not all depleted of colloid, lined by a high-cuboidal epithelium some of which is desquamated.

FIG. 6.  $\times 180$ , case 4. Specimen 12 hr. *post mortem* after 349 g. of thiouracil and 12 ml. of thyrotrophic extract. Similar picture to Fig. 5 but increased desquamation.

FIG. 7.  $\times 45$ , case 6. Specimen 20 hr. *post mortem* after 44 g. of thiouracil, formol-saline fixation. Large, medium-sized and small follicles. Irregularly distributed and varying in colloid content.

FIG. 8.  $\times 190$ , case 6. Specimen 20 hr. *post mortem* after 44 g. of thiouracil, formol-saline fixation. Vacuolated, cuboidal follicular epithelium.

## PLATE 3

FIG. 9.  $\times 190$ , case 1 (a). Specimen 47 hr. *post mortem*, 170 days after stopping a 336 g. course of thiouracil, formol-saline fixation. Large and medium-sized, colloid-containing follicles lined by a low cuboidal epithelium.

FIG. 10.  $\times 90$ , case 1 (a). Specimen 47 hr. *post mortem*, 170 days after stopping a 336 g. course of thiouracil, formol-saline fixation. Islet of small, round, empty follicles lined by a high-cuboidal epithelium.

FIG. 11.  $\times 275$ . Specimen 10 hr. *post mortem* of normal human thyroid after 7 ml. of thyrotrophic extract, formol-saline fixation. Follicles vary in size, many are depleted of colloid, and lined by a cuboidal epithelium.

# THE NEUROVASCULAR LINK BETWEEN THE NEUROHYPOPHYSIS AND ADENOHYPOPHYSIS

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There is little doubt that the secretory activity of the adenohypophysis is to some extent under the control of the nervous system [see Marshall, 1936, 1942; Brooks, 1939]. Two hypotheses have been advanced by various authors to explain this neural control: first, that the glandular cells possess a direct secretor-motor nerve supply, or secondly, that a humoral relay transmits the nervous stimuli from the hypothalamus by means of the hypophysial portal vessels.

The nerve supply of the hypophysis is derived from several sources. A sympathetic supply was first described by Bourguery in 1845. It consists of a few fine twigs passing from the carotid plexus to the pars distalis. The method of termination of these fibres and their function remain doubtful. Possibly they end on gland cells and are secretomotor, but more probably they end on blood vessels and are vasomotor. It is certain, however, that they do not subserve many of the nervous influences acting on the adenohypophysis. For example, coitus still excites ovulation in the rabbit and pseudopregnancy in the rat [Vogt, 1931, 1933; Haterius, 1933] after cervical sympathectomy. Other autonomic pathways that have been suggested, such as a supply via the greater superficial petrosal nerve [Hinsey & Markee, 1933], have likewise been shown to be unessential for the ovulation response in the rabbit [Vogt, 1942].

The major nerve supply to the hypophysis arises in the hypothalamus and passes to the gland through the hypophysial stalk. This supply has been described in all vertebrates studied from cyclostomes to mammals [for references see Fisher, Ingram & Ranson, 1938]. However, the number of fibres which pass from the hypothalamico-hypophysial tract to the pars distalis is uncertain. Some workers have described numerous fibres passing from the neural lobe into the adenohypophysis [Truscott, 1944], possibly ending in pericellular nets around the glandular cells [Pines, 1925; Brooks & Gersh, 1941]. The majority of workers find these fibres to be few in number. Rasmussen [1938], after a careful study in a variety of forms, found large areas of the adenohypophysis to be free of nerve fibres.

The possibility of a neurohumoral transmission of stimuli has been tentatively suggested on many occasions since the classical work of Popa & Fielding [1930, 1933], in which they first described a system of portal vessels linking the hypothalamus with the hypophysis. Wislocki & King [1936] and Wislocki [1936, 1937] confirmed the presence of these vessels in the hypophysial stalk but differed in their views on the extent of the system and direction of blood flow in it. The descriptions of the systemic circulation of the hypophysis given by various authors are in more general agreement. The pars distalis is supplied by small branches (the superior hypophysial

arteries) derived from the internal carotid artery. These run caudo-ventrally (in most forms) into the anterolateral region of the gland. The venous drainage of the pars distalis is by short trunks into the surrounding dural venous sinuses, though in some cases communication with the basal vein would appear to be present [Wislocki, 1937]. The infundibular process receives its arterial supply posteriorly. Small twigs from both internal carotid arteries run medially, may anastomose to some extent in the midline, and enter the posterior pole of the gland as the inferior hypophysial arteries. The venous drainage of the infundibular process is into the adjacent venous sinuses. The blood systems of the pars distalis and infundibular process appear to be separate and independent, the pars intermedia being in circulatory association with the infundibular process. According to Stevens [1937], the pars tuberalis and pars distalis are the most vascular parts of the hypophysis, with the infundibular process and the pars intermedia being vascular in that order.

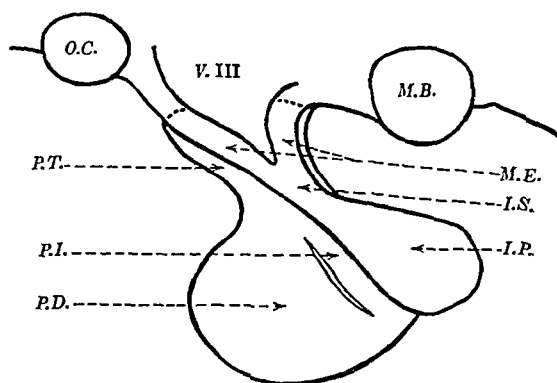


FIG. 1. Diagram to illustrate the terms used for the different parts of the hypophysis. The median eminence is differentiated from the hypothalamus proper. The neurohypophysis comprises: *M.E.* median eminence; *I.S.* infundibular stem; *I.P.* infundibular process; and the adenohypophysis: *P.T.* pars tuberalis; *P.I.* pars intermedia; *P.D.* pars distalis. (*M.B.* mammillary body; *O.C.* optic chiasma; *V. III*, third ventricle.)

The experimental evidence available indicates that neural control of the adenohypophysis is mediated in some way from the hypothalamus through the hypophysial stalk [Harris, 1937; Haterius & Derbyshire, 1937; Brooks, 1938; and others]. The present work is an investigation of the nervous and vascular connexions which may be involved in this transmission.

The terminology used for the various subdivisions of the hypophysis will be that proposed by Rioch, Wislocki & O'Leary [1939]. Since the clarity of the following account depends largely on the precision with which these terms are used, they are given below and illustrated in Fig. 1.

Adenohypophysis	Lobus glandularis	<div><div>Pars distalis (anterior lobe)</div><div>Pars tuberalis</div><div>Pars intermedia</div></div>	} Posterior lobe
Neurohypophysis	Lobus nervosus (neural lobe)	Infundibular process	
	Infundibulum (neural stalk)	<div><div>Infundibular stem</div><div>Median eminence of tuber cinereum</div></div>	

(Neural stalk + associated sheath of lobus glandularis = hypophysial stalk).

## HYPOTHALAMIC NERVE SUPPLY OF THE ADENOHYPOPHYSIS

*Material and methods*

The hypophyses of twelve rabbits, one monkey and one man have been studied. Only normal glands were used, except for the human gland which was removed and fixed within a few hours of death from pulmonary embolism. Of the twelve rabbit glands six were stained by de Castro's method. The remaining glands were stained by Bodian's method [Bodian, 1936] or by a variant based on Ungewitter's technique [Ungewitter, 1943]. This last method serves to intensify preparations otherwise poorly impregnated. The procedure was as follows. After protargol impregnation and reduction in Bodian's reducer, the sections were washed in warm running tap water for 5-10 min., washed 4-6 times in distilled water (1 min. each wash), and placed in 1% silver nitrate for 20 min. They were then rinsed in three changes of distilled water and reduced once more in Bodian's reducer (3 min.). If impregnation was still insufficient the intensifying process was repeated indefinitely until the desired result was obtained, care being taken to ensure uncontaminated solutions. When a satisfactory final stage was reached (black nerve fibres on a yellow background), the sections were either left untuned or toned in 0.3% gold chloride, fixed in hypo, washed and mounted.

*Observations*

Nerve fibres were seen in small numbers passing from the infundibular process into the pars intermedia (Pl. 1, fig. 3). These fibres are fine and tortuous. In the greater part of the pars distalis we could find no similar fibres, and have been unable to trace any nerve fibres through the pars intermedia into this part of the gland. Reference will be made later to nerve fibres associated with the pars tuberalis and the portal vascular system.

## VASCULAR CONNEXIONS BETWEEN THE NEUROHYPOPHYSIS AND ADENOHYPOPHYSIS

*Material and methods*

The vascular connexions have been studied in preparations following indian-ink injections. The injections were made into the aorta or brachiocephalic artery in six rats, three guinea-pigs, seven rabbits and eight dogs immediately after death. More complete injections were obtained by injecting a solution of 25-50% ink in distilled water without preliminary washing out of the vessels with saline. After injection the calvarium was removed and the head fixed in 10% formalin for 1 week. Following further dissection, decalcification was carried out in Jenkin's solution and the trimmed block consisting of hypothalamus, hypophysis and surrounding tissue embedded in celloidin. Sections were cut serially at 100-200  $\mu$ , and were usually lightly counterstained.

In addition to these indian-ink preparations, two adult human pituitary glands were removed complete with stalk, hypothalamus and adjacent meninges. These were serially sectioned in celloidin at 30  $\mu$  and stained with iron haematoxylin. A further human gland and attached stalk was sectioned in paraffin wax at 5  $\mu$  and stained by Mallory's method and the Bodian silver method.

Finally, the relationship of the vessels and nerve fibres in the hypophysial stalk was studied in Bodian preparations of rabbit and human material.

## Observations

*Indian-ink preparations of animal pituitaries*

The general filling of the glands with the ink showed some features of interest. The blood supply of the infundibular process and pars distalis appears to be separate, as shown by the independent filling of the vessels in these two parts of the gland in the less satisfactory injections. The vessels of the pars tuberalis and median eminence filled most easily and completely with ink. The pars distalis was not found to be well injected in any specimen that did not show good filling of the tuberal region. The anterior part of the pars distalis usually contained more injected material than its caudal extremity, giving the impression that the flow occurs in an anterior to postero-inferior direction.

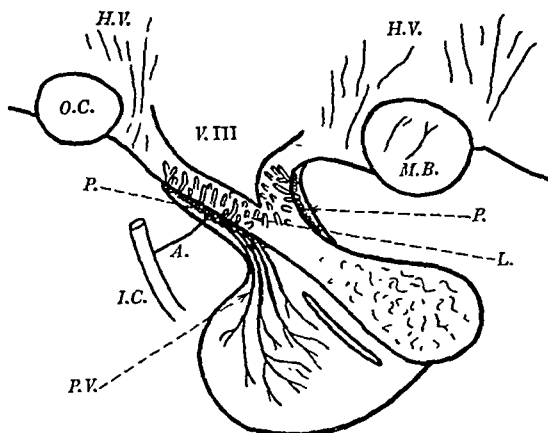


FIG. 2. Diagram of a sagittal section through the hypophysis and hypothalamus to show the general results. The arterial supply to the pars tuberalis is derived from small twigs (A.) from the internal carotid artery (I.C.). These twigs break up into a rich vascular plexus (P.) situated between the pars tuberalis and median eminence. From this plexus are derived vessels which enter the median eminence to form very characteristic sinusoidal loops (L.). These loops appear to drain into portal vessels (P.V.) which pass into the pars distalis and are distributed to the typical sinuses of this region. The vascular pattern of the median eminence is distinctly different from that of the hypothalamus (H.V.) and of the infundibular process. M.B. mammillary bodies; O.C. optic chiasma; V. III, infundibular recess of third ventricle.

The general results concerning the vascular pathway connecting the median eminence and the pars distalis are shown in Fig. 2. The blood supply of the pars tuberalis is derived from small branches of the internal carotid artery. These break up into several small twigs that penetrate the pars tuberalis to form a rich vascular plexus between the pars tuberalis and median eminence. (In the dog it extends down the infundibular stem, between the stem and the pars intermedia.) This large plexus nearly always injects well with ink. From the plexus arise vessels, in the form of sinusoidal loops, that enter the median eminence and infundibular stem and pass towards the infundibular recess of the third ventricle. In all the animals examined (rat, guinea-pig, rabbit and dog) we have noted these striking and characteristic loops in the median eminence. They also inject well with ink and are easily seen, appearing as long U-shaped capillary loops, either singly, branched, or occasionally as tufts arising from a single stem. Their length is variable, some penetrating only



a short distance into the neurohypophysis and others reaching almost to the end of the infundibular recess where terminal dilatations may be present. These loops remain separate and do not arborize one with another except in so far as a group may arise from a common parent vessel. An occasional loop may anastomose with one of the long, fine, hypothalamic vessels, and this has been seen most clearly at the anterior end of the median eminence of the dog. This is a rarity however, and we cannot agree with the conclusions of Basir [1932], that the capillary loops are drained by these anastomoses upwards to the tuber cinereum. The detailed pattern of the loops shows some variation in different animals. In the rat the vessels are most obvious as a midline tuft (Pl. 1, fig. 6) penetrating the median eminence. Since the hypophysial stalk of the rat passes almost horizontally backwards, the loops enter the median eminence in a vertical direction. In the guinea-pig (Pl. 1, figs. 4, 5), rabbit (Pl. 2, figs. 8-10) and dog (Pl. 2, figs. 12, 13), the loops are more widely distributed and more loosely arranged. They may be clearly seen in the anterior part of the median eminence where they are most numerous, in the lateral and posterior parts of this structure, and in the infundibular stem. They were also found in the central part of the infundibular process in the dog, in which animal the third ventricle verges upon this part of the gland. These latter vessels seem to arise from the plexus deep to the pars intermedia, though this could not be clearly confirmed for the most posterior loops as they appear to connect with the plexiform vessels of the neural lobe rather than with the plexus. A sharp distinction is thus visible between that part of the neurohypophysis which surrounds the infundibular recess and the remainder of the neural lobe, on the basis of their vascular pattern.

That the arterial supply of the loops is from the plexus underlying the pars tuberalis seems clear, but the drainage pathway is more difficult to trace in detail, for both afferent and efferent limbs pass into the region of this heavily injected vascular plexus. In some of our specimens, however, it is possible to trace individual loops and to observe them uniting into large trunks as they pass from pars tuberalis to pars distalis. In general it may be said that this region drains by large portal vessels passing postero-inferiorly into the pars distalis. This is well seen in a horizontal section through the median eminence of a rat hypophysis (Pl. 1, fig. 7). In the rabbit, the anterior part of the pars distalis (called by Dawson [1937] the zona tuberalis) is separated from the main bulk of the lobe by connective tissue trabeculae, which we have observed to be associated with the superior hypophysial arteries, branches of the internal carotid. This zona tuberalis of the rabbit, we think, may be regarded as a region of the adenohypophysis specialized to carry the vascular connexions from the median eminence and pars tuberalis to the main body of the pars distalis. It contains the numerous large trunks of the portal vessels, easily seen in both sagittal and horizontal sections (Pl. 2, figs. 8, 9, 11). In the dog the trunks of these vessels are not so clearly discernible, for the hypophysial stalk is foreshortened, and it appears that the middle trunks of the portal vessels are likewise abbreviated, leading to the approximation of the capillary plexuses at either end of the portal system.

#### *Human pituitary glands*

The general appearance of the portal system in the human material differs considerably from that seen in lower forms. Two facts, one technical and one anatomical,

partly explain this: first, the human specimens have not been injected with indian ink, and secondly, the greater length and different inclination of the hypophyseal stalk.

A striking feature of the human gland is the ease with which the portal vessels of the stalk may be seen with the naked eye, in either fresh or cleared specimens (Pl. 3, fig. 14). Serial sections through the hypothalamus, stalk, and gland show a very large number of blood vessels in the infundibular stem and median eminence (Pl. 3, fig. 15). These vessels are large, and, as described by Popa & Fielding [1930], possess well-defined sheaths. Inferiorly they pass to the anterior surface of the stalk and enter the pars distalis, partly via the pars tuberalis. Although these vessels have been seen passing forwards in profusion into the pars distalis they have not been traced into, or seen in, the infundibular process. In the infundibular stem they are tortuous but arranged mainly in a longitudinal plane. They appear to be formed in the stalk and median eminence by the union of clusters of smaller vessels, which are well seen in transverse sections (Pl. 3, fig. 16). The relationship between these clusters of smaller vessels and the loops seen in the indian-ink preparations of lower forms will be discussed later.

#### *Relationship of vessels to nerve fibres in the hypophyseal stalk*

Vessels and nerve fibres are probably related in a double manner in the hypophyseal stalk. First, in the neural tissue of the median eminence (and infundibular stem) the sinusoidal loops and tufts enter into intimate relationship with the great mass of nerve fibres present in this site (Pl. 3, figs. 16, 17). Secondly, nerve fibres have been seen in moderate number crossing from the hypothalamico-hypophyseal tract to the pars tuberalis in company with the capillary loops (Pl. 3, figs. 18, 19). It was not possible to confirm the termination of these fibres on glandular cells of the pars tuberalis (although this could not be excluded) but fibres have been seen which appeared to end on the blood vessels (Pl. 3, fig. 19). Several authors have described nerve fibres entering the pars tuberalis in a similar position [Cameron, 1929; Hair, 1938; Rasmussen, 1938], and Vasquez-Lopez [1942] drew attention to the association of nerve fibres and blood vessels in the narrow cortical zone of the median eminence and stalk, where this adjoins the pars tuberalis.

#### DISCUSSION

Our anatomical observations will be discussed first, followed by their possible functional significance.

Nerve fibres passing from the infundibular stem and process into the pars intermedia have been described by Gemelli [1906], Cajal [1911], Tello [1912], Croll [1928], Rasmussen [1938], Truscott [1944], and many others. The presence of these fibres we can confirm, although they appeared few in number. Hair [1938], Brooks & Gersh [1941], and Truscott [1944] were able to demonstrate a similar nerve supply for the pars distalis, although Hair (cat) and Brooks & Gersh (rat) admit that the number of fibres passing to the pars distalis is not large. Our results are in agreement with Rasmussen [1938], who was unable to find any convincing nerve supply to the glandular cells of the pars distalis. There are two technical difficulties in reaching a conclusion regarding the presence or absence of nerve fibres in the pars

distalis. First, impregnation of the nerve fibres with silver salts is often unsatisfactory. Croll [1928] suggested the reason might be failure of penetration of the fixative or silver salt, but found that increasing the time allowed for penetration did not improve the results. It might also be pointed out that even when thin ( $5\mu$ ) sections are stained on the slide, an increased number of stained nerve fibres does not appear. The second difficulty is the lack of a specific stain for nervous tissue, so that connective tissue may be confused with nerve fibres and pericellular nets. Although it is impossible to exclude, on anatomical data, a direct nervous influence of the hypothalamus on the secretion of the pars distalis, we feel this is sufficiently improbable to warrant investigation of alternative pathways.

The existence of a portal circulation of the hypophysis, as first described by Popa & Fielding [1930, 1933] for man, has been confirmed in a variety of animals. Regarding the precise extent of this system and the direction of blood flow within the portal vessels two views are current. On the one hand, Popa & Fielding maintain that the upper system of capillaries extends far into the hypothalamus, that the direction of flow in the vessels is upwards, and that the blood is collected by the lower system of capillaries from both lobes of the gland. On the other, Wislocki & King [1936] believe the upper limit of the portal system does not extend beyond the expanded lower end of the tuber cinereum (known as the median eminence), which is functionally part of the neurohypophysis. They claim the direction of blood flow to be downwards, all the blood entering the pars distalis. In general our results confirm those of Wislocki as to the extent of the portal system, the presence of a plexus in the pars tuberalis, and the presence of capillary loops or tufts in the median eminence and infundibular stem.

The vascular pattern of the median eminence varies in detail in different forms. In the rat the loops are concentrated in the midline anteriorly; in the dog discrete capillary loops are arranged more uniformly (as depicted by Wislocki [1937] for the cat), in the guinea-pig and rabbit the pattern is similar to that of the carnivores though the loops are less regular in shape, whilst in the monkey Wislocki & King [1936] described more branching of the vessels, which they call 'vascular tufts'. We have been unable to find a detailed account of human material injected with indian ink, but the microphotograph published by Wislocki [1936, Fig. 11] seems to show, as he stated, an arrangement similar to that of the monkey.

We shall not discuss here the problem of the direction of blood flow in the portal vessels, but one of us (J.D.G.) hopes to present the results of some experimental work on this subject at a later date.

It might be suggested that the pattern of the portal vessels is only an expression of the complex embryological development of the hypophysis, and lacks any underlying functional significance. This view would appear unlikely from a consideration of the relative vascularity of the hypothalamus, neural stalk and process. In our material one very striking feature was the great vascularity of the median eminence (and infundibular stem in man), as compared with adjacent neural tissue (Pl. 3, figs. 14, 15). The vascular nature of the median eminence of the rabbit is well shown in Pl. 2, fig. 10. It would seem that these vessels are more extensive than the nerve fibres and general neurohypophysial tissue require, so that they may have an additional function.

One hypothesis that has been tentatively suggested at intervals [Harris, 1937; Hinsey, 1937; Brooks, 1938; Taubenhause & Soskin, 1941] to explain the neural control of the adenohypophysis in the absence of a well-marked nerve supply is a humoral transmission of stimuli from the neurohypophysis to the pars distalis. The anatomical evidence available suggests that this transmission may occur from the median eminence through the portal system of vessels to the pars distalis. Some other evidence which has accumulated in favour of this view is summarized below.

(a) Ovulation in the rabbit normally occurs 10 hr. after coitus. If hypophysectomy is performed within 1 hr. of coitus ovulation does not occur, but if more than 1 hr. is allowed to elapse ovulation occurs normally [Fee & Parkes, 1929]. It seems that 1 hr. is necessary for the secretion of sufficient gonadotrophic hormone to produce follicular rupture. The explanation may be that stimulation of the hypophysis is normally effected by a slow humoral mechanism.

(b) Markee, Sawyer & Hollinshead [1946] have shown that ovulation in the rabbit is more easily elicited by electrical stimulation of the hypothalamus than of the hypophysis. One of us (G.W.H.) can confirm this in a limited number of rabbits stimulated in the conscious state by the remote-control method. (Further work is in progress.) If the adenohypophysis is devoid of a direct secretomotor nerve supply it is possibly not excited by direct electrical stimulation.

(c) The different effects recorded after section of the hypophysial stalk [Harris, 1937; Hinsey, 1937; Brooks, 1938] may be due to variations in regeneration of the portal vessels.

(d) The results of Dempsey [1939], Dey [1943], and Leininger & Ranson [1943], indicating that a greater disturbance of the oestrous cycle in the guinea-pig may follow a lesion in the median eminence than section of the hypophysial stalk, may be due to the former producing a complete irreparable denervation of the median eminence, whilst stalk section allows the possibility of vascular repair.

(e) In some animals—the whale, porpoise, sea-cow, armadillo, Indian elephant [Wislocki & Geiling, 1936; Geiling, Voss & Oldham, 1940; Oldham, McCleery & Geiling, 1938; Oldham, 1938; Wislocki, 1939]—the infundibular lobe is separated from the pars intermedia, or pars distalis if the intermedia is lacking, by a connective tissue septum derived from the capsule. This makes it difficult to visualize a neural pathway from the infundibular process to the pars distalis. However, from a study of the literature, it appears that these animals have a pars tuberalis in contact with the median eminence and probably a portal system of vessels.

(f) Taubenhause & Soskin [1941] state that the pars distalis of the rat may be stimulated by local application of a mixture of prostigmine and acetylcholine. They adduce this fact as evidence for the theory of humoral control of the adenohypophysis.

Sufficient evidence is not available to prove neurohumoral control of the adenohypophysis, but we feel this theory has much to support it.

It is of interest to speculate about the nervous pathways by which the hypothalamus might influence the capillary loops in the median eminence and infundibular stem, and so form the first link in a neurovascular chain. The nerve fibres entering the tuber cinereum, apparently destined to supply the neurohypophysis, have been described as arising in the following regions and nuclei in the hypothalamus: the

supraoptic nucleus, paraventricular nucleus, anterior nucleus, ventromedial nucleus, ventral periventricular nucleus, ventral hypothalamic nucleus pars centralis [Young, 1936], scattered cells in the tuberal region, and perhaps in the mammillary region [see Fisher *et al.* 1935; Ingram, 1939]. Lesions placed in the hypophyseal stalk or median eminence cause a clear-cut retrograde degeneration in only the supraoptic and paraventricular nuclei [Rasmussen, 1939; Frykman, 1942], but it is possible that some of the scattered cells and more loosely knit nuclei in the hypothalamus also undergo some degeneration. At the moment it cannot be decided which of the above nuclei, if any, are linked with the portal vessels. It is possible to state, however, that the capillary loops in the median eminence are surrounded by large numbers of nerve fibres derived from various hypothalamic nuclei, which in turn are connected with the thalamic and subthalamic centres, and so with the cerebral cortex and other regions of the nervous system.

As to the nature of the hypothetical humoral substance transmitted via the portal vessels there is little evidence. Two possibilities are the hormone(s) of the neurohypophysis (since the median eminence has been shown to be part of the secretory neurohypophysis [Magoun, Fisher & Ranson, 1939] and acetylcholine or related compounds. This latter suggestion receives some support from the work of Taubenhauß & Soskin [1941]. Excitation of the neurohypophysis probably entails a cholinergic mechanism at some point on the neural pathway [Pickford, 1939], thus making it difficult to obtain direct evidence of a similar mechanism for the adenohypophysis.

#### SUMMARY

1. The anatomy of the nervous and vascular connexions between the neurohypophysis and adenohypophysis is described.
2. The nervous connexions are scanty in the rabbit, monkey, and man.
3. The vascular connexions are prominent in the rat, guinea-pig, rabbit, dog and man. They are described with particular reference to the capillary loops found in the median eminence and infundibular stem, and the hypophyseal portal vessels.
4. Nerve fibres from the hypothalamico-hypophyseal tract are intimately associated with the capillary loops.
5. It is suggested that the central nervous system regulates the activity of the adenohypophysis by means of a humoral relay through the hypophyseal portal vessels.

It is a pleasure to record our thanks to Prof. H. A. Harris for his ever-willing advice and encouragement, to Dr F. W. Gunz for his help in obtaining human material, to Mr J. A. F. Fozzard for his skilful microphotography, and also to Messrs J. Cash and R. Smith for their valuable aid in the preparation of the histological material.

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## EXPLANATION OF PLATE 1

FIG. 3. Photomicrograph of a horizontal section through the pituitary gland of a rabbit. Note the relative scarcity of nerve fibres passing from infundibular process (*I.P.*) to pars intermedia (*P.I.*). Bodian stain.  $\times 250$ .

FIG. 4. Photomicrograph of a sagittal section through the median eminence and adjacent structures of a guinea-pig. Blood vessels injected with indian ink. *M.E.* median eminence containing sinusoidal loops; *P.D.* pars distalis; *P.T.* pars tuberalis; *V. III*, third ventricle.  $\times 30$ .

FIG. 5. Photomicrograph of a horizontal section through the pituitary gland of a guinea-pig. Blood vessels injected with indian ink. *A.* arterial twigs supplying the pars tuberalis (*P.T.*) and its vascular plexus; *I.P.* infundibular process; *M.E.* median eminence; *P.D.* pars distalis.  $\times 16$ .

FIG. 6. Photomicrograph of a transverse section through the median eminence of a rat. Blood vessels injected with indian ink. *B.* base of skull and meninges; *H.* hypothalamus; *M.E.* median eminence showing the tuft of capillary loops; *P.T.* pars tuberalis; *V.* third ventricle.  $\times 56$ .

FIG. 7. Photomicrograph of a horizontal section through the hypothalamus and pituitary gland of a rat. Blood vessels injected with indian ink. Note the small arterial twigs (*A.*) which supply the pars tuberalis (*P.T.*) and its associated vascular plexus, and thus the loops of the median eminence. The portal vessels (*P.V.*) are easily seen running from the pars tuberalis and median eminence, along the ventral surface of the infundibular stem, to the pars distalis (*P.D.*).  $\times 19$ .

## EXPLANATION OF PLATE 2

FIG. 8. Photomicrograph of a sagittal section through the hypothalamus, pituitary gland and related parts of the skull of a rabbit. Blood vessels injected with indian ink. *H.* hypothalamus; *I.P.* infundibular process; *M.B.* mammillary bodies; *O.C.* optic chiasma; *P.D.* pars distalis.  $\times 7$ .

FIG. 9. Higher power view of the median eminence shown in Fig. 8. *H.* hypothalamus; *I.S.* infundibular stem; *L.* loops in median eminence; *P.T.* pars tuberalis; *P.V.* portal vessels in zona tuberalis (part of pars distalis).  $\times 30$ .

FIG. 10. Photomicrograph of an oblique section through the median eminence of a rabbit. (Plane of section shown by line *A-A* in Fig. 8.) Blood vessels injected with indian ink. *M.E.* median eminence containing a large number of the typical sinusoidal loops, and surrounded by a mantle of pars tuberalis (*P.T.*) with its associated vascular plexus; *P.D.* pars distalis.  $\times 19$ .

FIG. 11. Photomicrograph of an oblique section through the zona tuberalis and pars distalis of a rabbit. (Plane of section shown by line *B-B* in Fig. 8.) Blood vessels injected with indian ink. *P.D.* pars distalis; *Z.T.* zona tuberalis (a specialized part of the pars distalis of the rabbit, continuous superiorly with the pars tuberalis) containing the trunks of the portal vessels.  $\times 19$ .

FIG. 12. Photomicrograph of a sagittal section through the hypothalamus, pituitary gland and related structures of a dog. Blood vessels injected with indian ink. *H.* hypothalamus; *I.P.* infundibular process; *M.E.* median eminence with contained sinusoidal loops; *P.D.* pars distalis; *V. III*, third ventricle.  $\times 15$ .

FIG. 13. Photomicrograph of a sagittal section through the median eminence of a dog. Blood vessels injected with indian ink. *M.E.* median eminence containing characteristic vessels; *P.D.* pars distalis; *V. III*, third ventricle.  $\times 56$ .

## EXPLANATION OF PLATE 3

FIG. 14. Photograph of hypothalamus, stalk, and pituitary gland of a man. Posterior view. *M.B.* mammillary bodies; *O.T.* optic tract; *P.* pituitary gland; *P.S.* pituitary stalk, posterior aspect, showing portal vessels engorged with blood.  $\times 4$ .

FIG. 15. Photomicrograph of a sagittal section through hypothalamus and pituitary gland of a man. Iron haematoxylin stain. *H.* hypothalamus; *I.P.* infundibular process; *I.S.* infundibular stem showing a large number of capillaries and portal vessels; *O.C.* optic chiasma; *P.D.* pars distalis; *V. III*, third ventricle. Note the portal vessels passing from the convexity of the infundibular stem into the pars distalis.  $\times 4$ .

FIG. 16. Photomicrograph of a transverse section through the hypophyseal stalk of a man (Bodian stain) to show the clusters of small vessels which unite to form the portal trunks. *P.T.* pars tuberalis on surface of infundibular stem.  $\times 30$ .

FIG. 17. Photomicrograph of a cluster of small vessels in the human hypophyseal stalk (Bodian stain) to show the intimate relation between these vessels and the nerve fibres of the hypothalamico-hypophyseal tract.  $\times 250$ .

FIGS. 18, 19. Different regions of a sagittal section through the median eminence of a rabbit (Bodian stain) to show nerve fibres passing from the hypothalamico-hypophyseal tract (*H.T.*) into the pars tuberalis (*P.T.*) in intimate relation with the blood vessels (*V.*). Both  $\times 437$ .

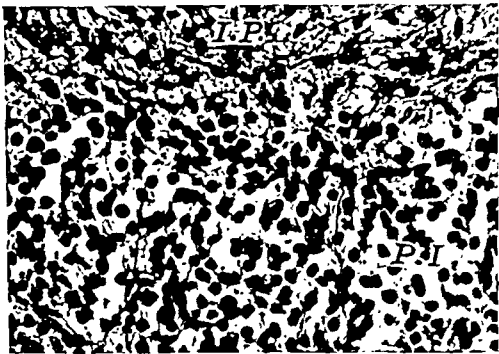


FIG. 3.  $\times 250$ .

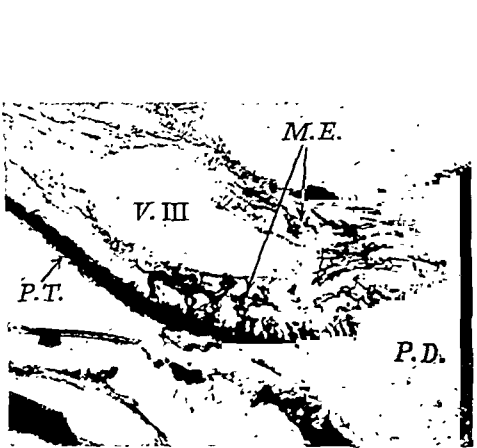


FIG. 4.  $\times 30$ .

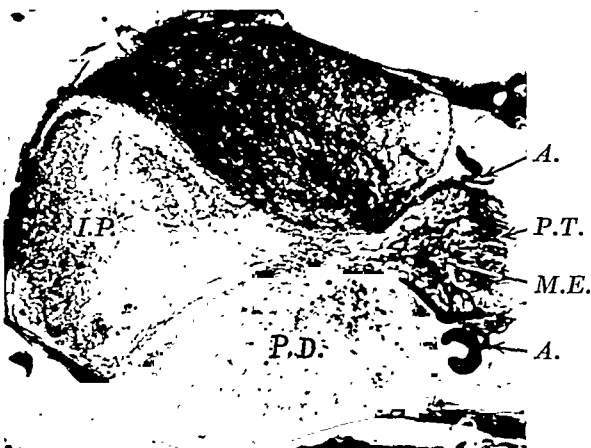


FIG. 5.  $\times 16$ .



FIG. 6.  $\times 56$ .

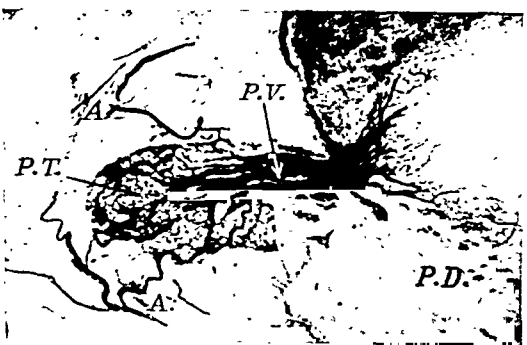


FIG. 7.  $\times 19$ .



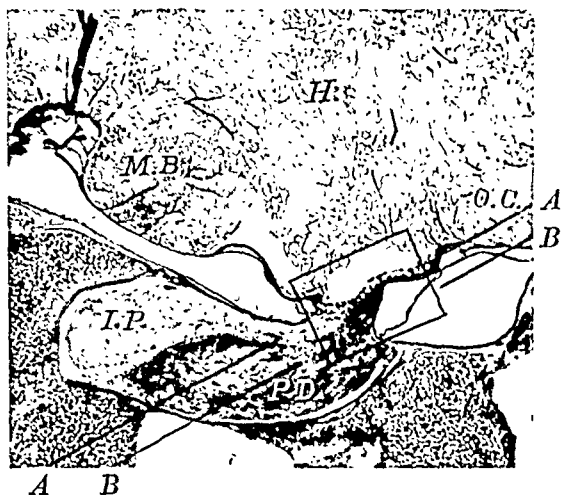


FIG. 8.  $\times 7$ .

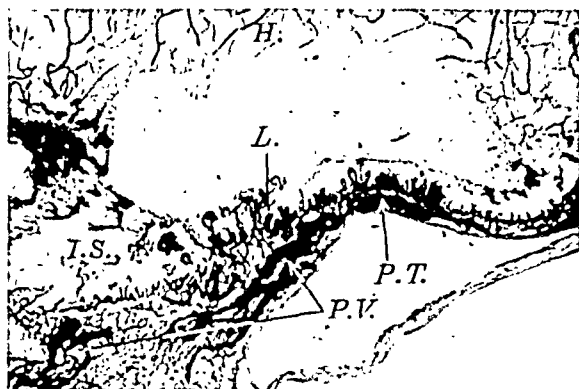


FIG. 9.  $\times 30$ .



FIG. 10.  $\times 19$ .



FIG. 11.  $\times 19$ .

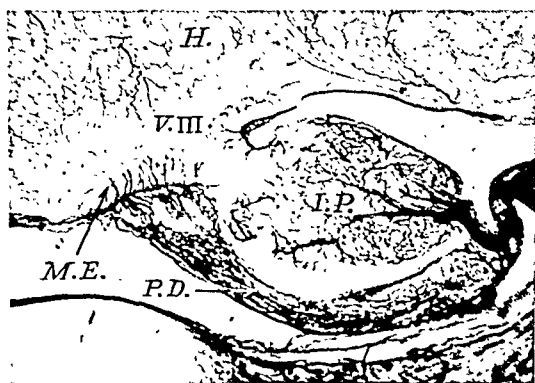


FIG. 12.  $\times 15$ .



FIG. 13.  $\times 50$ .

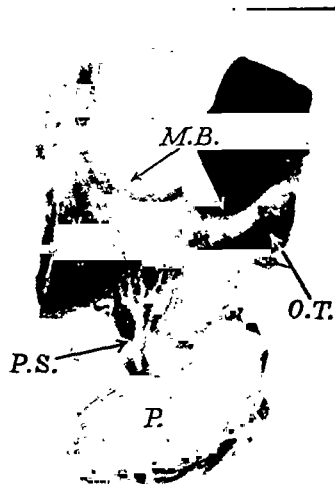


FIG. 14.  $\times 4$ .

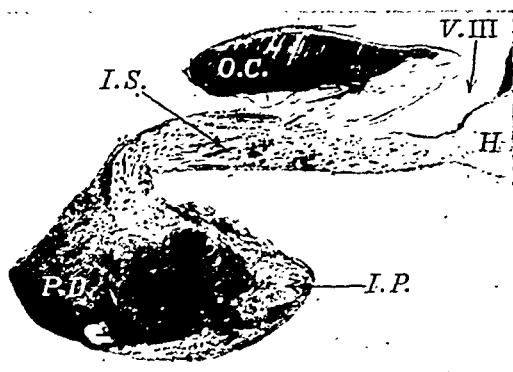


FIG. 15.  $\times 4$ .

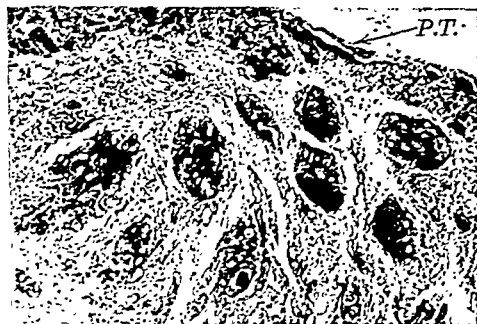


FIG. 16.  $\times 30$ .

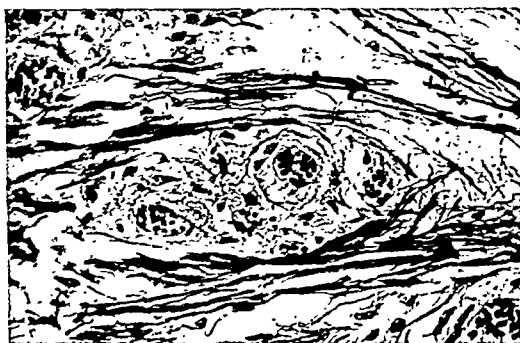


FIG. 17.  $\times 250$ .



FIG. 18.  $\times 437$ .

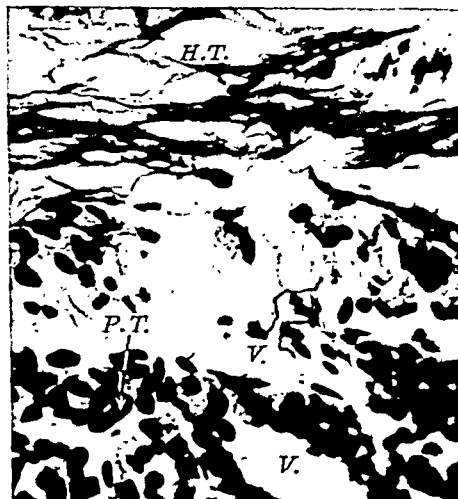


FIG. 19.  $\times 437$ .



# LEANNESS IN ADRENALECTOMIZED RATS

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In an earlier paper [Tuerkischer & Wertheimer, 1942] it was shown that adrenalectomized rats show a gradual depletion of their peripheral fat depots which is quite independent of their general condition. It was possible that the depletion of the fat reserves was due, in part or in full, to a reduced food intake. In the present investigation, therefore, the loss of depot fat in adrenalectomized rats is compared with that in paired-fed, sham-operated animals. In further experiments the role of the adrenal cortex hormones in the maintenance of fat depots is evaluated. A recent comprehensive review of the literature on the relationship between the adrenal cortex and fat metabolism has been given by Ingle [1943].

## METHODS

Male albino rats (100–120 g.) from the laboratory stock were used. The animals were kept chiefly on a diet of wheat with the addition of bread and vegetables. The 'high-carbohydrate diet' consisted of starch 80 % and casein 20 %; the 'high-fat diet' of margarine 55 %, casein 35 % and starch 10 %; and the 'high-protein diet' of casein 70 %, carbohydrates 20 % and fat 10 %. The usual supplements of salt and vitamins were added. Rubin-Krick solution was given in place of drinking water. Two days after adrenalectomy controlled feeding was started. Groups of sham-operated animals were paired-fed with each adrenalectomized group. The peripheral fat fraction (mixed renal, mesenteric, testicular and groin fat) was carefully separated and weighed. It was then returned to the decapitated carcass from which the alimentary tract had been removed. Nitrogen was determined by Kjeldahl's method and glycogen by a micro-modification of the method of Pflüger [Good, Kramer & Somogy, 1933]. Liver fat and total fat were determined by extraction in alcohol and ether (two treatments with each solvent). Elaidic acid was administered in the form proposed by Sinclair [1935], elaidin representing 40 % of the total caloric intake. Its determination in the adipose tissue was carried out by Sinclair's method [1935]. Iodine number was determined according to the method of Rosenmund & Kuhnhehn [1923].

## RESULTS

### *Fat loss in adrenalectomized rats*

In adrenalectomized rats the peripheral fat reserve was considerably smaller than in paired-fed controls. In summer the peripheral fat depots were completely depleted in most cases within a fortnight of the operation; in winter the reaction was even more rapid. The loss of other fat was also considerable, but less marked as comparison of total-fat and peripheral-fat values shows (Table 1).

Table 1. *Fat deposition in adrenalectomized rats on standard diet.*  
*all values in g./100 g. body weight*

Animals	No. of rats	Days of exp.	Changes in body weight	Weight of		Liver weight	Liver fat	Liver protein*
				Peripheral fat	Total body fat			
Adrenalectomized	14	14	$-5.5 \pm 1.3$	$0.79 \pm 0.19$	$2.95 \pm 0.51$	$3.23 \pm 0.10$	$0.13 \pm 0.01$	$0.63 \pm 0.01$
Controls	14	14	$+5.3 \pm 2.0$	$2.84 \pm 0.24$	$5.37 \pm 0.63$	$3.51 \pm 0.13$	$0.16 \pm 0.01$	$0.70 \pm 0.03$

\* Mean of 8 experiments

The loss of fat after adrenalectomy was independent of the general condition of the animal. It was uniformly observed in animals which died with a sudden drop of body-temperature, but was also present in animals which were killed in good condition. The loss was also independent of body-weight changes, being seen both in the adrenalectomized animals whose body-weight changes were identical with those of the paired-fed controls and in animals whose weight deviated from the latter in any direction. The loss of peripheral fat was also independent of the nature of the diet. In Table 2 typical experiments showing marked fat loss after adrenalectomy on high-carbohydrate, high-protein, and high-fat diets are listed.

Table 2. *Fat deposition in adrenalectomized rats on different diets:*  
*all values in g./100 g. body weight*

Animals	Diet	Days of exp.	Changes in body weight	Weight of		Liver fat	Liver protein
				Peripheral fat	Total body fat		
Adrenalectomized	High-carbohydrate	19	+36	3.55	6.50	3.88	0.20
Control	High-carbohydrate	19	+47	7.27	11.70	3.93	0.27
Adrenalectomized	High-fat	13	-2	0.10	1.61	4.00	0.21
Control	High-fat	13	-3	3.19	6.17	4.70	0.24
Adrenalectomized	High-protein	16	0	0.10	—	3.97	0.19
Control	High-protein	16	+11	2.70	—	4.60	0.28

In medullectomized animals there was no loss of fat.

Adrenalectomized animals did not differ significantly from paired-fed controls in their liver weight. The fat and nitrogen contents of the liver were slightly lower and liver glycogen levels were uniformly lower in the adrenalectomized rats than in the paired-fed control rats.

#### *Influence of adrenal cortex hormones on fat reserves of adrenalectomized rats*

Adrenalectomized rats which received daily 2 mg. of deoxycorticosterone acetate (Doca) showed a fat reserve which was considerably higher than that of adrenalectomized rats which were paired-fed with them and given injection of 2 ml. of oil only (Table 3). In a few experiments with adrenalectomized rats daily deoxycorticosterone acetate injections were started 14 days after operation (when the fat depots should be largely depleted) and continued for a further 14 days. It was found that the fat loss of the rats at the end of this time was considerably smaller than in untreated adrenalectomized animals; but it is emphasized that we have not so far

been able by administration of 2 mg. of deoxycorticosterone acetate daily for 2 weeks to increase the fat reserves of normal rats beyond the level observed in paired-fed controls. In a second series, concentrated adrenal cortex extract (Kendall, 1 ml.  $\equiv$  150 g. of fresh adrenal gland) was given in place of the steroid. The effect in this case was less marked.

Table 3. *Fat deposition of adrenalectomized rats treated with adrenal cortex hormones: all values in g./100 g. body weight*

Treatment	No. of rats	Days of exp.	Weight of peripheral fat	Liver weight	Liver fat
Doca	10	19	$1.98 \pm 0.35$	$3.95 \pm 0.12$	$0.19 \pm 0.01$
	10	19	$0.60 \pm 0.18$	$3.11 \pm 0.10$	$0.15 \pm 0.01$
Whole cortex extract	6	13	$1.68 \pm 0.45$	$3.65 \pm 0.05$	$0.17 \pm 0.006$
	6	13	$0.85 \pm 0.41$	$3.50 \pm 0.12$	$0.14 \pm 0.01$

*Deposition of dietary fat in fat depots of adrenalectomized rats*

The process of deposition of dietary fat in depots has been investigated with the help of elaidin according to the method of Sinclair [1935]. A typical experiment is represented in Table 4.

Table 4. *Deposition of elaidic acid in peripheral fat depots of adrenalectomized rats: all values in g./100 g. body weight*

Animal	Days of exp.	Amount of ingested food containing elaidin	Weight of peripheral fat	Solid acids	Iodine no.
Adrenalectomized	6	36	3.3	0.275	30.7
Adrenalectomized	6	29	2.2	0.208	25.9
Control	5	28	3.9	0.243	31.8
Control without elaidin	—	—	—	0.206	7.6

#### DISCUSSION

Loss of body fat can be due to any of the following causes.

(1) Increase of total metabolic rate. Adrenalectomized rats, however, show a diminished total metabolic rate.

(2) Increase of fat metabolism at the cost of a decrease in carbohydrate metabolism. Adrenalectomized rats, however, show the reverse behaviour.

(3) Diminution of fat absorption in the alimentary tract. The majority of investigators have, however, abandoned the view that fat absorption is decreased after adrenalectomy [Ingle, 1943]. Determinations of faecal fat in normal and adrenalectomized rats maintained on a high-fat diet (55 % fat) have been made by us, and the same amounts of faecal fat were found in normal and adrenalectomized animals.

(4) Enhancement of depot-fat mobilization. Adrenalectomy markedly delays or prevents the induction of fatty liver by phosphorus poisoning [Verzar & Laszt, 1935] or pituitary extract [MacKay & Barnes, 1937; Fry, 1937]. Different investigators have with some justice inferred from this that the adrenalectomized animal experiences difficulty in mobilizing depot fat. The possibility is present, however, that the experimental results are due rather to an inability on the part of the liver to store fat after adrenalectomy. There is no support for the view that the liver of adrenalectomized animals is capable of metabolizing fat at an augmented rate. It is probable,

on the contrary, that liver-fat metabolism is decreased by adrenalectomy. There is, in any case, no experimental proof that the leanness of adrenalectomized animals is due to an enhanced mobilization of depot fat.

(5) Decreased deposition of peripheral fat. Experiments on deposition of elaidic acid in adipose tissue show that adrenalectomized animals are capable of incorporating elaidic acid into their adipose tissues. The evidence of these experiments does not support the view that adrenalectomy leads to a decrease in fat deposition. The amounts of elaidic acid found by us in the adipose tissue of the adrenalectomized animals did not differ markedly from those found in the controls. It is difficult, however, to draw quantitative conclusions concerning total fat deposition from the available data.

(6) Impairment of carbohydrate-fat conversion. We have earlier emphasized [Tuerkischer & Wertheimer, 1942] the probability that the conversion of carbohydrate into fat is depressed by adrenalectomy. The present experiments with rats on high-fat diet (55 % fat) in which the fat loss is especially marked show, however, that other factors also influence the results. The adrenalectomized animals maintained on a diet of extremely high fat content and entirely lacking in carbohydrate suffered from marked loss of appetite and died in a short time.

The position is therefore that no explanation for our findings can be offered at present. The manner of the transfer of dietary fat to the adipose depots needs re-investigation with the help of the Schoenheimer technique. The possibility of a disturbed fat neogenesis from carbohydrate should be studied further by appropriate gas metabolism measurements. According to recent results [MacKay, Wick & Barnum, 1946] utilization of acetone bodies in fasting rats is increased following adrenalectomy. The level of the acetone bodies, however, remains constant, and it may be inferred therefore that ketogenesis in this condition is increased. The results of MacKay *et al.* [1946] are valid, however, only for fasting rats. In any case re-examination of the manner of the utilization of fat by adrenalectomized rats should be undertaken.

Samuels & Conant [1944] concluded from experiments on mobilization of stored fat that adrenalectomized animals use up their depot fat at an abnormally slow rate. In the experiments made by these authors the rats were kept for 6 days on a diet of pure fat and then starved for 4–6 days. It is evident that the conditions employed differ considerably from our own, where care was taken to maintain an optimal intake of food. Adrenalectomized rats which are starved for a considerable period enter into a depressed state, characterized by diminished basal metabolism and low body temperature. The interpretation of the results of Samuels & Conant is made somewhat difficult by this circumstance.

#### SUMMARY

1. Adrenalectomized rats when compared with paired-fed, sham-operated rats show an extensive loss of body fat. The fat loss following adrenalectomy is independent of the general condition, the course of the body-weight change, and the nature of the diet.

2. Daily administration of 2 mg. of deoxycorticosterone acetate to adrenalectomized rats completely prevents the depletion of the fat depots. Whole adrenal cortex extract is less effective in this respect.

3. Tracer experiments with elaidic acid failed to prove that the rate of the deposition of dietary fat in the adipose tissue is reduced by adrenalectomy.
4. The influences which govern the depletion of the fat depots are discussed.

This research was aided by a grant from the Dazian Foundation for Medical Research. We are much indebted to the Ciba Pharmaceutical Products for the supply of deoxycorticosterone acetate and to Dr E. C. Kendall for his gift of a concentrated adrenal cortex extract.

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# THE INSTABILITY OF OESTROGENS IN SOLUTION

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The loss of oestrogenic potency in aqueous solutions of dienoestrol and stilboestrol has already been reported by us [Wilder Smith & Williams, 1945], while the chemical instability of similar solutions was demonstrated independently by Warren & Gulden [1945]. The present paper describes the examination of some of the conditions affecting the stability of these and other oestrogens in solution.

## METHODS

### *Preparation of solutions*

All solutions were prepared by dissolving 10 mg. of the oestrogen in 100 ml. of absolute alcohol or acetone. Aliquot portions of these solutions were diluted with the solvent to be investigated to give 1  $\mu\text{g./ml.}$ , and dilutions of these stock solutions were made at intervals for biological testing. A trace of toluene was added to the aqueous and urinary solutions to prevent bacterial or mould contamination. All except the urinary solutions were kept at room temperature.

### *Biological assay*

The routine method used in this Institute was followed. Sprayed rats were injected with 0.5 ml. of solution twice daily for 3 days, and their vaginæ were smeared twice daily for the two following days. Smears consisting entirely of cornified and/or epithelial cells were regarded as positive and the results expressed on a percentage basis.

Since the sensitivity of the colony varies from time to time, an accurate assay of the activity of any solution involves testing it at three dose levels on groups of ten to twenty rats and at the same time similarly testing a freshly prepared solution of the oestrogen: concurrent experiments with a large number of different solutions would require many hundreds of rats weekly, so we have carried out such assays only on solutions which were relatively *stable*. The percentage activity remaining in an old solution was assessed from the colony regression line for the oestrogen by Gaddum's method of calculation as described by Burn [1937]. Standard errors including two results out of three are given in parentheses after each such percentage value.

The *unstable* solutions were tested periodically on groups of ten rats, usually until a 500% overdose gave a negative response. Such tests have little statistical value, and though instability may be clearly shown, differences in rates of deterioration are only roughly indicated.

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The dose:response relations for fresh solutions of the five oestrogens obtained during the course of the present experiments are given in Table 1 so that experimental values given in text or tables may be assessed.

Table 1. *Normal dose:response relations of freshly prepared oestrogen solutions*

Dose μg.	Oestrone (127)* Response (%)	Dienoestrol (200) Response (%)	Stilboestrol (228) Response (%)	Hexoestrol (93) Response (%)	Oestradiol (77) Response (%)
2.0	83	—	—	—	—
1.5	53	—	—	—	—
0.9	10	85	—	—	—
0.7	—	29	—	—	—
0.5	—	20	81	94	—
0.4	—	—	52	60	—
0.3	—	—	17	15	—
0.2	—	—	—	—	81
0.1	—	—	—	—	24

\* Total number of rats used is given in parentheses.

## RESULTS

### *Solutions in water*

In aqueous solution (1 μg./ml.) hexoestrol, oestrone, and oestradiol are relatively stable. Solutions which were assayed after standing in the laboratory for 2–4 months showed only slight loss of activity of doubtful significance (Table 2).

Table 2. *Stability of relatively stable oestrogens in aqueous solution (1 μg./ml.)*

Solute	Age of solution (weeks)	Oestrogenic activity remaining (%)	Limits of error (%)
Hexoestrol	24	85	75–97
Hexoestrol + H <sub>2</sub> O <sub>2</sub>	9	83	73–94
Oestrone	27	94	70–127
Oestradiol	28	69	53–90

Dienoestrol and stilboestrol, on the other hand, were unstable in aqueous solutions. Some of the factors that might influence the instability were investigated.

### *Effect of pH and oxidation*

The figures in Table 3 show the deterioration in oestrogenic activity of aqueous solutions of dienoestrol containing 1 μg./ml. The rate of decay is accelerated when dienoestrol is dissolved in acid (N/500 HCl) or alkali (N/500 NaOH), or by aeration of the solution. Solutions of dienoestrol were prepared and divided into two parts and air was bubbled through one for an hour; the aerated portion lost activity more rapidly than the control portion. This is demonstrated in two separate experiments in Table 3. As might be expected from this last finding the rate of deterioration is also hastened in the presence of hydrogen peroxide (0.2 ml. of 100 vol. hydrogen peroxide per 500 ml. of solution), and benzoyl peroxide is equally effective in this respect. The control aqueous solutions of dienoestrol used in these experiments were prepared at different times. Their rates of deterioration were not the same in all cases, but the differences were not great and could be attributed to the inaccuracy of the tests.

Table 3. *Instability of aqueous dienoeostrol solutions*

Age of solution (weeks)	Dose* ( $\mu\text{g.}$ )	Oestrous response in spayed rats (%)			
		Solvent: Water	Acid	Alkali	$\text{H}_2\text{O}_2$
0	0.5	26	11	56	0
1	1.0	35	5	0	—
	3.0	—	—	—	0
2	3.0	100	0	0	—
3	3.0	100	—	—	—
	6.0	—	0	0	—
8	3.0	0	—	—	—
		Aerated water		Aerated water	
		Solvent: Water		Water	
1	1.5	100	90	100	100
4	2.0	100	0	100	0
6	3.0	100	—	100	—
	6.0	—	0	—	0
12	2.0	0	—	—	—

Bracketed experiments were carried out simultaneously.

\* Doses of dienoeostrol originally present in solution.

Aqueous stilboestrol solutions showed much wider variations. Old solutions of stilboestrol prepared in exactly similar fashion but at different times may still give a 30 % response with a 0.5  $\mu\text{g.}$  dose after 7 weeks, or at the other extreme may give a negative response with a dose of 1.0  $\mu\text{g.}$  after 2 weeks. This variation was also observed in the effect of oxidizing agents. The more stable of the solutions cited above, for example, was the control portion of a solution other portions of which were aerated or had benzoyl peroxide added. The aeration had no detectable effect in 7 weeks, and the benzoyl peroxide only produced a hastened deterioration between the 5th and 7th weeks (20 % response to a dose of 1.0  $\mu\text{g.}$  at the latter time). This result was, however, exceptional, and in other experiments the acceleration of the rate of decay by both these procedures or by the addition of hydrogen peroxide was very clear. We conclude that stilboestrol is unstable in aqueous solutions containing 1  $\mu\text{g./ml.}$ , and that while the instability is normally greater in the presence of oxidizing agents other factors are also concerned.

The effects of hydrogen peroxide were investigated on the relatively stable oestrogens hexoestrol and oestrone. There was no deterioration in the biological activity of the hexoestrol solution (see Table 2), but some loss of activity was produced in the aqueous oestrone solution of which a dose of 3.0  $\mu\text{g.}$  was quite inactive after 9 weeks. The production of a relatively inactive lactone by the action of hydrogen peroxide on oestrone has been described by Westerfeld [1942].

#### *Effect of concentration*

An alcoholic solution of stilboestrol (0.01 %) was diluted with water to form solutions containing 1, 2, or 3  $\mu\text{g./ml.}$  When tested 12 weeks later at doses equivalent to 0.7  $\mu\text{g.}$  of the originally added stilboestrol the responses were 10, 20 and 80 %; this difference between the three solutions had been consistently shown in two intermediate tests. Less concentrated solutions are apparently more unstable than stronger ones, as would be expected.

*Effect of light and temperature*

When aqueous solutions of stilboestrol and dienioestrol (1  $\mu\text{g.}/\text{ml.}$ ) were kept at 5° C. in the dark they still lost activity, though the rate of loss appeared to be reduced.

*Attempts at stabilization*

The foregoing experiments suggested that aqueous solutions of dienioestrol and stilboestrol might retain their biological activity in the presence of anti-oxidants. Attempts to maintain the potency of dilute solutions by keeping them under nitrogen were not uniformly successful. Better stabilization was, however, obtained by the addition of hydroquinone.

An aqueous solution of dienioestrol (1  $\mu\text{g.}/\text{ml.}$ ) was made up in the usual manner with the addition of 1  $\mu\text{g.}$  of hydroquinone per ml. Activity was lost at first, and 1.5  $\mu\text{g.}$  were required to produce a response of 40 % after 6 weeks. Thenceforth, however, there was little or no further loss and doses of 1.5 and 2.0  $\mu\text{g.}$  gave responses of 20 and 90 % 21 weeks later. Assays carried out when the solution was 17 and 27 weeks old showed that 51 % (42–64 %) and 45 % (39–54 %) of the original activity remained.

A similar solution of stilboestrol in water with added hydroquinone showed 69 % (58–83 %) and 65 % (58–74 %) of the original activity to be present when it was assayed after standing for 17 and 29 weeks. A control portion of the same solution without added hydroquinone gave a negative response to a dose of 3.0  $\mu\text{g.}$  after 11 weeks, indicating a loss of more than 85 % of the original activity. It is clear that hydroquinone has a definite stabilizing effect.

Table 4. *Stability of oestrogens in sesame oil solution (1  $\mu\text{g.}/\text{ml.}$ )*

Solute	Age of solution (weeks)	Oestrogenic activity remaining (%)	Limits of error (%)
Dienoestrol	32	70	59–83
Stilboestrol	32	87	76–98
Hexoestrol	18	86	76–96
Oestradiol	16	110	88–138
Oestrone	34	89	71–110

*Solutions in sesame oil*

Solutions of all five oestrogens prepared in sesame oil at the same concentration as the aqueous solutions (1  $\mu\text{g.}/\text{ml.}$ ) were apparently stable (Table 4).

*Solutions in alcohol and acetone*

The routine initial step in preparing oestrogen solutions in this laboratory is to dissolve 10 mg. in 100 ml. of alcohol or acetone. Hence the importance of investigating the stability of such solutions.

The figures given in Table 5 show that under these conditions oestrone, oestradiol and hexoestrol are stable while dienioestrol is not. Stilboestrol is more stable here than in aqueous solutions: from the figures it appears to occupy an intermediate

position between the three stable oestrogens and dienioestrol. An alcoholic solution of dienioestrol with added hydroquinone elicited an 80 % response with a dose of 0.5  $\mu$ g. after standing 9 months, indicating no loss of activity in this time.

Table 5. *Stability of oestrogen solutions in alcohol or acetone (100  $\mu$ g./ml.)*

Oestrogen	Solvent	Age in weeks	Result of assay or test
Oestrone	Alcohol	18	102 % (84–111 %)
	Acetone	7	1.0 $\mu$ g. gives 11 % response
Dienioestrol	Alcohol	15	21 % (17–25 %)
	Acetone	12	3.0 $\mu$ g. gives 0 % response
Stilboestrol	Alcohol	17	73 % (63–84 %)
	Acetone	9	0.6 $\mu$ g. gives 100 % response
Hexoestrol	Alcohol	17	0.5 $\mu$ g. gives 100 % response
Oestradiol	Alcohol	17	0.2 $\mu$ g. gives 100 % response

#### *Solutions in urine*

When alcoholic solutions of the five oestrogens were diluted with normal men's urine to form solutions containing 10 mg./l., all except hexoestrol lost activity. At least part of this loss was due to adsorption of the solute from these supersaturated solutions on to the walls of their glass containers. This was proved when the bottles were well shaken, emptied and washed out with boiling acetone when 10–20 % of the originally added oestrogen was found in the acetone.

When more dilute solutions (1 mg./l.) were studied all were stable when kept in the refrigerator. This is shown by the results given in Table 6. The slight loss of activity in the dienioestrol solution was attributed to its being left at room temperature for 10 days. This was confirmed when part of another dienioestrol solution (1 mg./l.) in urine that had been kept at 5° C. was exposed to room temperature; this part lost activity while the part remaining at 5° C. did not.

Table 6. *Stability of oestrogens in men's urine (1  $\mu$ g./ml. kept at 5° C.)*

Oestrogen	Age of solution (weeks)	Dose given ( $\mu$ g.)	Response (%)
Oestradiol	16	0.15	40
Stilboestrol	12	0.35	33
	18	0.40	33
Hexoestrol*	26	0.50	89
Dienioestrol†	11	0.80	0
	—	1.00	67
Oestrone	12	1.00	100
	22	0.80	100

\* 10 mg./l.

† Kept at room temperature for 10 days.

When urine solutions (1 mg./l.) of the five oestrogens were kept in the refrigerator for very long periods (6 months or longer) some loss was usually apparent except in the case of hexoestrol.

#### COMMENT

##### *Instability in laboratory solvents*

We are well aware of the quantitative limitations of our experiments, but our object has been purely practical and we feel justified in drawing the following conclusions.

Aqueous solutions of dienoestrol or stilboestrol (1  $\mu\text{g./ml.}$ ) are unstable and should be prepared freshly, both for biological assays and for experimental work. More concentrated solutions (100  $\mu\text{g./ml.}$ ) in alcohol or acetone behave in the same way. Oily solutions of these two oestrogens, however, are apparently stable enough to be prepared and left for several weeks without appreciable loss of potency, and all the solutions of the other three oestrogens we have examined have shown a similar stability. At the same time it is advisable that even the solutions which we regard as stable should as a normal precaution be tested from time to time against freshly prepared solutions.

These are the practical conclusions. Any attempt to explain the mechanism of the instability or the apparent stabilization after 50 % of the activity has been lost in aqueous solutions with added hydroquinone would require more accurate quantitative data than we have time or facilities to provide. Chemical examination of an old alcoholic solution of dienoestrol showed that a proportion of a carboxylic acid was present, so that the loss of oestrogenic activity is probably due to chemical breakdown as well as to polymerization, as we earlier suggested.

#### *Stability in urine*

Had the oestrogens proved unstable in urinary solutions obvious difficulties would have arisen in accurate determinations of oestrogen excretion in clinical practice or metabolism experiments. Fortunately we find that provided the usual precautions are taken with urine specimens no loss of activity occurs. The reasons for this stabilization do not lie in the maintenance of low temperature only, since aqueous solutions are not stabilized thus. It is probable that the explanation lies in the anti-oxidant action of the polyphenols present.

#### SUMMARY

The stability of solutions of oestrone, dienoestrol, stilboestrol, hexoestrol and oestradiol has been tested.

Dienoestrol and stilboestrol lose oestrogenic potency when kept in aqueous solution (1  $\mu\text{g./ml.}$ ) or in solution in alcohol or acetone (100  $\mu\text{g./ml.}$ ). Oestrone, hexoestrol, and oestradiol are relatively stable under the same conditions.

The instability of dienoestrol and stilboestrol is greater when the solutions are aerated or in the presence of benzoyl peroxide or hydrogen peroxide. The loss of activity may be partially inhibited in the presence of hydroquinone.

All five oestrogens are relatively stable in dilute solution (1  $\mu\text{g./ml.}$ ) in sesame oil.

All these oestrogens were relatively stable in solution in normal men's urine (1 mg./l.) kept at low temperature.

We are very grateful to the Council of the Middlesex Hospital Medical School for providing our laboratory facilities and to the Medical Research Council for a grant for our assistants Miss D. D. Andrews and Mr B. D. Shepherd whose help has been invaluable.

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# THE RELATION BETWEEN DIRECT AND INDIRECT OESTROGENIC FUNCTION IN A SERIES OF SYNTHETIC OESTROGENS

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*(Received 1 January 1947)*

The exhibition of oestrogen in the experimental animal is followed by a number of effects which vary with the dose. Certain of these, the proliferatory changes in the vagina [Sotiriadou, 1940], uterus and nipples [Lyons & Pencharz, 1936; Chamberlin, Gardner & Allen, 1941], are direct effects, whilst others, those on mammary development [Gomez, Turner & Reece, 1937] and on lactation [Turner, 1939], which are mediated through the anterior pituitary gland, may be regarded as indirect effects. Diethylstilboestrol is one of the many synthetic compounds of widely different formulae which have oestrogenic activity of a high order [Dodds, Lawson & Noble, 1938]. This compound has been shown to promote mammary development [Dodds *et al.* 1938] and to have effects on lactation, stimulatory or inhibitory, depending on the dose [Folley & Scott Watson, 1938; Folley, 1941], and, indeed, differs functionally from the naturally occurring oestrogens only in a few minor respects. To determine whether modification of the diethylstilboestrol structure results in compounds with different ratios of direct to indirect oestrogenic activity is the object of this investigation. The chemical description and structural formulae of the six compounds which have been compared with diethylstilboestrol are shown in Table 1.

## DIRECT OESTROGENIC ACTIVITY

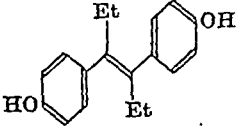
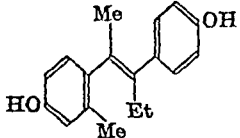
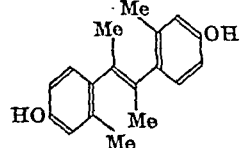
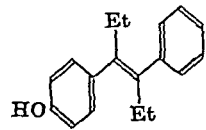
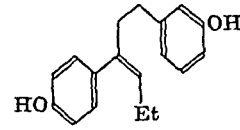
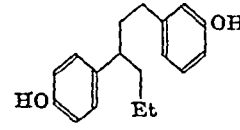
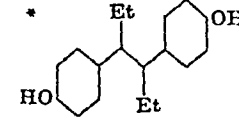
### *Method*

Direct oestrogenic activity was estimated by measuring the increase in the weight of the uteri of immature spayed rats using essentially the same method as that described by Bülbring & Burn [1935]. Rats of inbred Wistar strain, weighing 30–35 g., were ovariectomized and left for a period of 1 week before treatment with the drugs, which were given on three successive days in a total of 0.6 ml. of arachis oil in the case of subcutaneous treatment, and of 1.5 ml. of 2.5% gum acacia in the case of oral administration.

### *Results*

The comparison of direct oestrogenic activity of the seven synthetic compounds by subcutaneous and oral routes revealed two interesting facts. The first is that, while the slopes of the lines relating the logarithms of the dose to the uterine-weight responses are the same for lower dose ranges, at higher doses the curves are probably different as shown by a simple statistic relating the differences between means and their respective standard errors (Fig. 1). Also the slopes of the lines for the different routes of administration for the same compound are different (Figs. 1, 2). We associate these differences with variation in the rates of absorption and metabolism and in differences in

Table 1. *A comparison of the direct oestrogenic activities of the synthetic oestrogens determined by increase in the uterine weight of spayed immature rats*

Compound		Activity compared with that of diethylstilboestrol; limits of error ( $P=0.95$ ) in parentheses	
		By subcutaneous injection in oil	By stomach tube in suspension
4:4'-Dihydroxy- $\alpha$ : $\beta$ -diethylstilbene, m.p. 171° (diethylstilboestrol)		1	1
4:4'-Dihydroxy- $\beta$ -ethyl-2: $\alpha$ - dimethylstilbene, m.p. 153-4°		1.2 (0.7-2.2)	4.8 (0.6-38)
4:4'-Dihydroxy- $\alpha$ : $\beta$ :2:2'-tetra- methylstilbene, m.p. 208-11° [Hudson & Walton, 1946]		1.5 (0.9-2.5)	6.2 (2.5-15.0)
4-Hydroxy- $\alpha$ : $\beta$ -diethylstilbene, m.p. 125-7° [Brownlee, Copp, Duffin & Tonkin, 1943]		0.025 (0.006-0.1)	0.017 (0.005-0.06)
1-( <i>m</i> -Hydroxyphenyl)- 3-( <i>p</i> -hydroxyphenyl)- $\Delta^3$ (or $\Delta^2$ )- <i>n</i> -hexene (resinous) [Hudson, 1946]		<0.001	—
1-( <i>m</i> -Hydroxyphenyl)-3- ( <i>p</i> -hydroxyphenyl)- <i>n</i> -hexane (resinous) [Hudson, 1946]		<0.001	—
Perhydro-(4:4'-dihydroxy- $\alpha$ : $\beta$ - diethylstilbene), m.p. 188°	* 	0.0017 (0.0008-0.004)	—

\* Assumed structure.



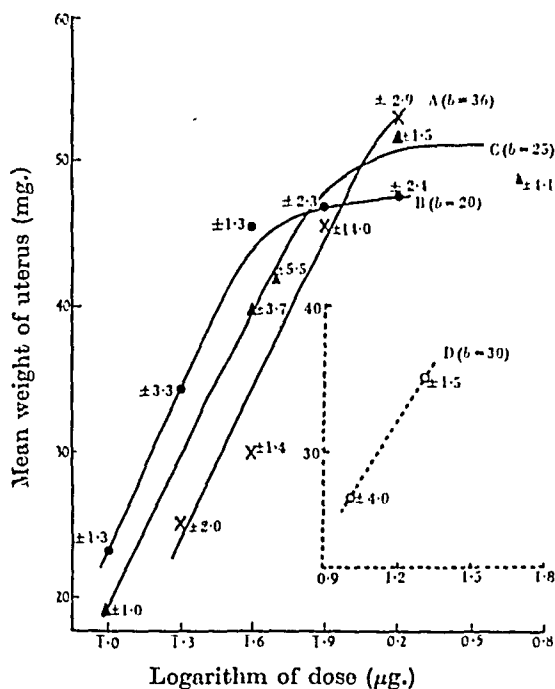


FIG. 1. The curves relating the logarithms of the doses of synthetic oestrogens injected subcutaneously to the mean weights of uteri of rats, together with the standard errors corresponding to the ordinates, and (b) the calculated slopes of the lines. (A) Diethylstilboestrol; (B) 4:4'-dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene; (C) 4:4'-dihydroxy- $\beta$ -ethyl- $\alpha$ :2-dimethylstilbene; and (D) 4-hydroxy- $\alpha$ : $\beta$ -diethylstilbene.

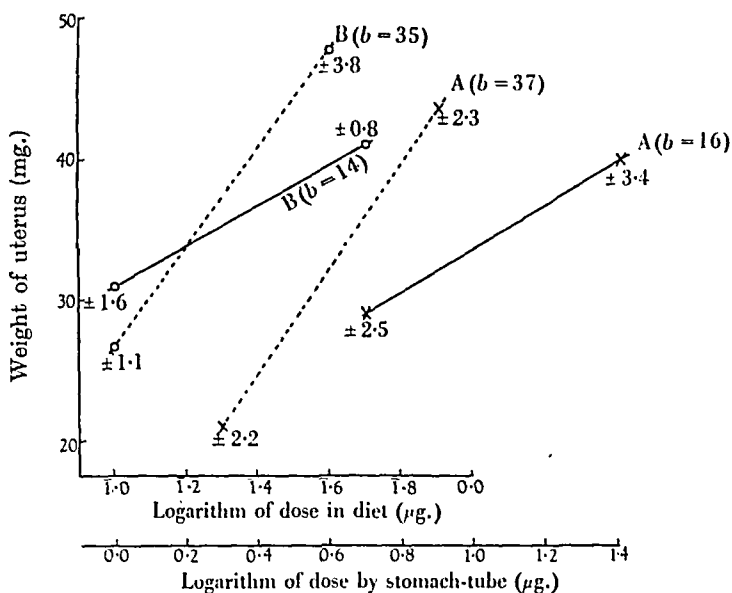


FIG. 2. The curves relating the logarithm of the doses of synthetic oestrogens, given by stomach-tube and by incorporation in the diet, to the mean weight of the uteri of rats. The intact lines relate to oestrogens by stomach-tube; the broken lines to oestrogens in the diet. A is diethylstilboestrol; B is 4:4'-dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene.

the partition between oily solutions and body fluids. In one instance shortage of material dictated the use of only two points on the curve. The results, together with the limits of error corresponding to  $P = 0.95$ , are presented in Table 1, in the form of a ratio compared with stilboestrol and are derived by calculation from equations fitted to four calculated regression lines. Data are given in Fig. 1.

One compound, 4:4'-dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene (tetramethylstilboestrol), possesses very striking oestrogenic properties, being of similar activity to diethylstilboestrol when compared by the subcutaneous route and some six times as active when administered daily as a suspension by stomach-tube. Because of the high order of activity of tetramethylstilboestrol by the latter route, it is of interest to compare its activity with diethylstilboestrol when both compounds are incorporated in the diet of the experimental animals. The results, which are summarized in Fig. 2, show that smaller doses of either oestrogen were required to produce the same effect on the uterus, and that, whereas tetramethylstilboestrol is some six times as active as diethylstilboestrol when treatment is given daily by stomach-tube, the difference is at most only threefold when the compounds are incorporated in the diet. These observations may well be explained by the rapid emptying of the stomach contents of the rat [Grollman, 1941] and the more ready absorption of tetramethylstilboestrol.

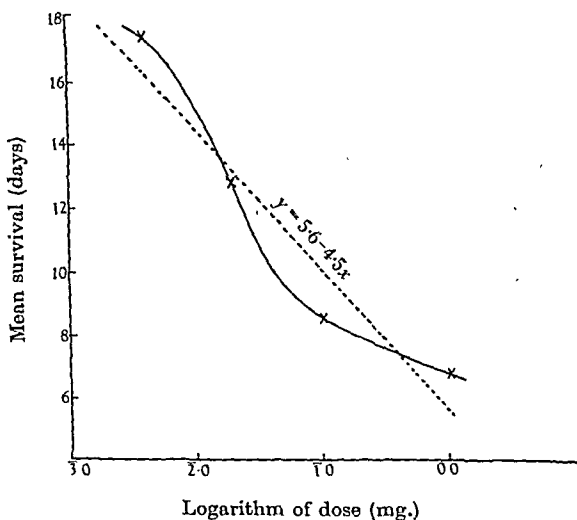


FIG. 3. Curve relating the logarithm of the dose of diethylstilboestrol injected subcutaneously in the lactating rat, to the mean survival (days) of the litter.

#### INDIRECT OESTROGENIC ACTIVITY

##### Method

Indirect oestrogenic activity was measured by comparing the efficiency of the compounds in inhibiting lactation in the parturient rat as reflected by rate of growth and mortality in the litter [Folley & Kon, 1938]. Groups of three or four rats weighing 160–200 g., and with the number of young in each litter as far as possible adjusted to eight, were treated with the compounds from the 3rd day *post partum* until the 21st day or until all the pups had died. The weights of the pups and the mortality were recorded daily. A smooth curve (Fig. 3) has been found to relate the logarithm of the

dose of diethylstilboestrol injected subcutaneously to the mean survival days calculated from three to four litters each of eight pups.

In experiments involving the use of ovariectomized rats, the ovaries were removed under tribromethanol anaesthesia within 12 hr. of parturition.

At death of the litter or on the 21st post partum day the pituitaries, mammae, ovaries, and adrenals of the mother rat were removed for histological examination.

### *Results*

The comparative efficiencies of the seven synthetic compounds, assessed by reference to a regression line fitted to the standard curve (Fig. 3), are reproduced in the form of ratios (together with the limits of error corresponding to  $P=0.95$ ) in the last columns of Tables 2 and 3; these reproduce the protocols of experiments in which the routes of administration were subcutaneous and oral respectively. Only those compounds possessing direct oestrogenic activity suppressed lactation in the parturient rat.

#### PATHOLOGICAL CHANGES PRODUCED IN THE ANTERIOR PITUITARY, OTHER GLANDS AND SECONDARY SEX ORGANS

Variation in the normal histological picture of the anterior pituitary, adrenal, and ovarian glands (and to a lesser extent in the uterus) of the parturient, lactating Wistar rat is, in our experience, very marked. Administration of 'direct' oestrogenic substances in doses large enough to inhibit lactation, to however slight an extent, results in changes well outwith the normal variations which are encountered, and amount to gross damage. They caused: in the pars glandularis loss of granules from almost all the cells, hypertrophy by increase in the number of reserve cells and a subsequent decrease in the proportion of eosinophil cells; in the uterus gross hypertrophy with convolution of the epithelium and vacuolation of the mucosa; in the mammae loss of active secretory tissue; in the adrenal cortex congestion, vacuolation, and loss of trabecular pattern and in extreme cases vacuolation and congestion in the adrenal medulla.

### DISCUSSION

The effects of oestrogen in promoting mammary development and in regulating lactation are mediated through the anterior pituitary gland [Turner, 1939]. We have classified these as indirect effects, as opposed to the direct oestrogenic effects which result in proliferation of the uterus, cornification of the vagina, and nipple growth. In the present series of compounds related to diethylstilboestrol it has not been possible to dissociate direct from indirect oestrogenic function. On the contrary, it seems that a simple ratio relates the ability to increase the weight of the uterus in the immature ovariectomized rat to the activity in suppressing lactation in the adult rat (compare Table 1 with the last column in Tables 2 and 3), for it seems likely that any small differences which are apparent are due to the conditions of the tests and the varying rates of absorption and elimination of the compounds, reflected in the differences in slopes of the log dose: uterine-weight response curves. The similitude of action of each of the present series of compounds is also exhibited by their greatly reduced efficacy in suppressing lactation in the spayed animal (Table 4) and by the similarity of the damage they cause in the pituitary, adrenals, mammae and uterus of the lactating rat. The reduced effect in the spayed animal provides evidence that the drug action in

Table 2. *A comparison of the effects of daily subcutaneous injections of the synthetic oestrogens on the mean weights of lactating rats and on the weights and survival of the litters*

Compound	Dose (mg.)	No. of pups	No. of litters	Mean % variation in body weight on the 10th day post partum		% mortality of pups on 21st day	Mean survival of pups; maximum = 18 (days $\pm$ s.e.)	Activity compared with that of diethylstilboestrol; limits of error ( $P=0.95$ ) in parentheses
				Doe	Pups			
4:4'-Dihydroxy- $\alpha$ : $\beta$ -diethylstilbene (diethylstilboestrol)	0.004	21	3	+0.5	+172	19	17.5 $\pm$ 0.3	1
	0.02	21	3	0.0	+158	67	12.8 $\pm$ 0.9	
	0.1	22	3	-7.1	+100	100	8.5 $\pm$ 0.5	
	1.0	33	4	-6.5	+69.5	100	6.6 $\pm$ 0.5	
4:4'-Dihydroxy- $\beta$ -ethyl-2: $\alpha$ -dimethylstilbene	1.0	28	3	-5.3	+85.4	100	7.5 $\pm$ 0.3	0.36 (0.15-0.8)
4:4'-Dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene	1.0	26	3	-6.4	+62.0	100	6.3 $\pm$ 0.5	0.7 (0.27-1.8)
4-Hydroxy- $\alpha$ : $\beta$ -diethylstilbene	1.0	29	3	-4.0	+98	90	11.0 $\pm$ 0.6	0.063 (0.028-0.14)
1-( <i>m</i> -Hydroxyphenyl)-3-( <i>p</i> -hydroxyphenyl)- $\Delta^3$ (or $\Delta^2$ )- <i>n</i> -hexeno	1.0	20	2	+2.0	+175	10	17.6 $\pm$ 0.4	Inactive
1-( <i>m</i> -Hydroxyphenyl)-3-( <i>p</i> -hydroxyphenyl)- <i>n</i> -hexano	1.0	32	3	+4.7	+172	37	16.0 $\pm$ 0.7	Inactive
Perhydro (4:4'-dihydroxy- $\alpha$ : $\beta$ -diethylstilbene)	0.1	22	3	+5.5	+184	4.0	17.5 $\pm$ 0.5	Inactive
Controls (arachis oil, 0.1 ml.)	—	34	3	+4.6	+181	3.0	17.9 $\pm$ 0.1	—

Table 3. *A comparison of the effects of synthetic oestrogens given daily by mouth on the mean weights of the lactating rats and on the weights and survival of the litters*

Compound	Dose (mg.)	No. of pups	No. of litters	Mean % variation in body weight on the 10th day post partum		% mortality of pups on 21st day	Mean survival of pups; maximum = 18 (days $\pm$ s.e.)	Activity compared with that of diethylstilboestrol; limits of error ( $P=0.95$ ) in parentheses
				Doe	Pups			
4:4'-Dihydroxy- $\alpha$ : $\beta$ -diethylstilbene (diethylstilboestrol)	1.0	23	3	-7.0	+103.1	74	10.4 $\pm$ 1.1	1
4:4'-Dihydroxy- $\beta$ -ethyl-2: $\alpha$ -dimethylstilbene	1.0	26	3	-5.3	+91.8	100	8.6 $\pm$ 0.5	2.5 (0.68-9)
4:4'-Dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene	1.0	27	3	-6.2	+164	100	7.2 $\pm$ 0.5	5.2 (1.4-19)
4-Hydroxy- $\alpha$ : $\beta$ -diethylstilbene	1.0	25	3	-3.2	+156.3	32	16.3 $\pm$ 1.1	0.049 (0.013-0.18)
Controls (arachis oil, 0.1 ml.)	—	23	3	+5.3	+159	4.3	17.3 $\pm$ 0.7	—

the intact animal is not due to a toxæmia in either the mother or the pups resulting from secretion of the substance with the milk—as has been suggested by Meites & Turner [1942].

Table 4. *A comparison of the effects on the mean weights of two different series of ovariectomized lactating rats receiving synthetic oestrogens, one series by subcutaneous injection and one by mouth, and of the effect on the weights and survival of the litters. The last column gives the corresponding figures for intact lactating rats derived from Tables 2 and 3*

Compound	Dose (mg.)	Mean % variation in body weight on the 10th day <i>post partum</i>		Mortality of pups on 21st day	Mean survival of pups with spayed mothers; maximum = 18 (days $\pm$ s.e.)	Mean survival of pups with intact mothers; maximum = 18 (days $\pm$ s.e.)
		Doe	Pups			
Compounds given by subcutaneous injection						
4:4'-Dihydroxy- $\alpha$ : $\beta$ -diethylstilbene (diethylstilboestrol)	1	- 12.5	+ 130	2/9	15.9 $\pm$ 1.5	6.6 $\pm$ 0.5
4:4'-Dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene	1	- 5.7	+ 180	7/7	14.1 $\pm$ 0.5	6.3 $\pm$ 5.0
Controls		0	+ 240	1/8	17.8 $\pm$ 0.3	17.9 $\pm$ 0.1
Compounds given by mouth						
4:4'-Dihydroxy- $\alpha$ : $\beta$ -diethylstilbene (diethylstilboestrol)	1.0	- 8.9	+ 126	5/7	11.0 $\pm$ 1.9	10.4 $\pm$ 1.1
4:4'-Dihydroxy- $\beta$ -ethyl-2: $\alpha$ -dimethylstilbene	1.0	- 2	+ 140	0/7	18.0 $\pm$ 0.0	8.6 $\pm$ 0.5
4:4'-Dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene	1.0	- 16.0	+ 100	8/8	12.0 $\pm$ 1.0	7.4 $\pm$ 0.5
4-Hydroxy- $\alpha$ : $\beta$ -diethylstilbene	1.0	- 3	+ 146	0/7 and 1/6	17.6 $\pm$ 0.3	16.3 $\pm$ 1.1
Controls (arachis oil, 0.1 ml.)		0	+ 200	1/3	16 $\pm$ 2.0	17.3 $\pm$ 0.7

The comparison of direct oestrogenic function by relating increase in uterine weights with the logarithms of the doses reveals the interesting fact that the slopes of these curves differ among themselves. For this reason the primary oestrogenic function is only to be assessed after the curve has been fully investigated and may not be derived from an isolated estimate fitted to an assumed curve. This finding also illuminates the qualitative differences between the compounds and underlines the fallacy of referring to 'activity' of oestrogens in an unqualified sense. The difference in the slopes occurs with both subcutaneous and oral administration, and as might be expected is seen in the examination of a single compound by both routes.

Two of the new compounds show striking oestrogenic activity compared with stilboestrol when given by mouth. The more active compound, 4:4'-dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene [Hudson & Walton, 1946], has the same order of activity as stilboestrol when injected in oil, but is some six times as effective when administered by stomach-tube. Of similar activity, 4:4'-dihydroxy- $\beta$ -ethyl-2: $\alpha$ -dimethylstilbene is rather more active than stilboestrol by subcutaneous comparison but some five times as effective when administered by stomach-tube. When the comparison is made with the

tetramethyl compound incorporated in the diet the efficiency of the new compound drops to threefold that of stilboestrol, an effect which may be associated with the rapid stomach-emptying time of rodents [Grollman, 1941].

## SUMMARY

1. The oestrogenic activities of a new series of synthetic compounds related to diethylstilboestrol have been assessed by their effect on the uterine weights of immature ovariectomized rats and by their efficiency in suppressing lactation in parturient rats.

2. In the present series of compounds the relative physiological efficiency is the same whether tested on the uterus or by suppression of lactation.

3. The effects of these compounds on lactation are greatly diminished when the mother is ovariectomized.

4. A similitude of effect and of an intensity varying with oestrogenic activity occurs in the anterior pituitary, adrenals, ovaries, uteri, and mammae of lactating rats under treatment, as viewed by the histological changes found.

5. The slope of log dose: uterine-weight curves for different oestrogens given by the same route varies, and provides evidence of varying rates of absorption and elimination.

6. These compounds show considerable difference in comparative activities by different routes. One compound (4:4'-dihydroxy- $\alpha$ : $\beta$ :2:2'-tetramethylstilbene) of similar activity by subcutaneous injection in oil is five to six times as active as diethylstilboestrol by mouth and three times as active when incorporated in the diet of the rats. An asymmetrical isomer of diethylstilboestrol (4:4'-dihydroxy- $\beta$ -ethyl-2: $\alpha$ -dimethylstilbene) has similar activities.

We are indebted to our chemical colleagues for supplies of the compounds shown in Table 1. Dr E. Walton and Mr B. J. F. Hudson, of the Wellcome Chemical Works, synthesized compounds 2, 3, 5 and 6, and Dr W. M. Duffin, of the Wellcome Chemical Research Laboratories, synthesized compounds 4 and 7. The histological preparations, and some aspects of the pathological report, were by Dr C. L. Oakley and Dr D. Trevan. We would also like to acknowledge the help of Mr B. Kent who was responsible for a number of the tests.

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# IMPLANTS OF SYNTHETIC OESTROGENS IN THE UDDERS OF SHEEP

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Although it is well established that oestrogens will induce lactation in virgin goats and maiden heifers [Folley, Scott Watson & Bottomley, 1940, 1941*a, b*; Lewis & Turner, 1940, 1941; Walker & Stanley, 1940, 1941; Folley, Stewart & Young, 1944; Folley & Malpress, 1944; Hammond & Day, 1944] there is still some controversy about how these substances cause udder growth in farm animals. Turner and his colleagues [Lewis & Turner, 1939; Mixner, Lewis & Turner, 1940; Trentin, Mixner & Lewis 1941; Trentin, Lewis, Bergman & Turner, 1943] conclude from their numerous experiments that ovarian hormones act by stimulating in the pituitary gland the formation or release of specific mammogenic hormones: mammogen I is stimulated by oestrogen and induces the development of ducts and canals, while mammogen II is under the control of progesterone and stimulates alveolar development. Evidence against this theory has, however, been advanced. For instance Lyons & Sako [1940] and Nelson [1941] found that local application of oestrogens to some of the mammae of castrated male or female guinea-pigs caused growth in the treated glands only, and similar findings have been reported by Speert [1940] and Gardner & Chamberlin [1941]. Further, Chamorro [1940, 1943] and Gardner [1941] produced growth of the mammary glands in hypophysectomized animals treated with oestrogen.

In the experiments reported here we have compared the effects of implants of synthetic oestrogen tablets made under the skin in the neck or directly in the udder of virgin sheep. We have measured the relative activities of the two means of administration by recording the period elapsing between the implantation and the onset of the induced lactation.

## METHODS

### *Animals*

The experimental animals were virgin sheep born in the spring of 1946. The experiment started in the autumn of the same year when the animals were 5-6 months old and weighed 25-30 kg.; the udders showed no development at this time. Throughout the experiment the animals were maintained on the same diet and in the same stables.

Daily examination of the animals was started 1 week after the implantation of the oestrogen tablets. The udder was examined for lactation, glandular development and teat growth. The animals were milked once daily (9 a.m.) until the daily yield reached 100 ml. and thereafter twice daily (morning and evening). The day on which the yield definitely started to rise (see accompanying graphs) was taken as the day of the onset of lactation, and there was no difficulty in determining it in this way.

The experiment was continued for 2-3 months and the animals were then sold to save expense. By this time the onset of lactation had been determined with certainty, and in some cases the peak of lactation had been reached.

### *Oestrogen administration*

All the tablets used contained 50 mg. of stilboestrol, and the twenty-one animals were divided into five experimental groups which received 1-3 of these tablets implanted in the neck or in the udder. Where the implantation was in the udder, it was always made in the right half. An incision was made in the skin of the udder and the tablet or tablets introduced into a small pocket. The wound was closed with clips, no damage having been done to the glandular tissue.

### RESULTS

The treatment given to the five experimental groups and the speed of onset of the lactation obtained are recorded in Table 1. The graphs in Figs. 1 and 2 show the actual milk yields of some of the sheep in the two most important groups.

It is evident that the onset of lactation was much quicker and more regular in the sheep implanted with one tablet in the udder (group 2) than it was in the sheep receiving three tablets subcutaneously in the neck (group 1). The difference cannot be attributed to the difference of dose, as when one tablet was implanted in the neck only one of the three sheep so treated came into lactation at all (group 3). The results also indicate that when two or three tablets were implanted into the udder the response was not so good as when one tablet was implanted; the onset of lactation in these cases differed little from that induced by the implantation in the neck.

In none of the animals implanted in the udder was there any visible difference in the growth of the two halves of the udder, nor any measurable difference in their milk yields.

Consideration of the graphs in Figs. 1 and 2 shows that the peak of lactation was attained earlier with implants in the udder than with implants in the neck. The lactation obtained was quite as good as we have previously obtained with neck implants and satisfactory for the season and age of the animals. Although we stopped the experiment 2-3 months after the implantations had been made, at this time the milk yields in the neck-implanted sheep were still lower than those reached by udder-implanted sheep after 1 month.

### DISCUSSION

Among our experimental animals the most satisfactory results were obtained by the implantation of one 50 mg. tablet of stilboestrol in the udder. This mode of administration produced a faster and more regular onset of lactation than did implantation in the neck. This advantage is particularly clear when groups receiving the same dose of oestrogen are compared—for example, groups 2 and 3. The udder-implanted animals also showed a better development and growth of the glandular tissue and teats. We conclude that the oestrogen exerts a direct effect on the udder, local implantation producing a greater concentration at this point and minimizing the inactivation that will occur in the liver when the oestrogen is given elsewhere. How the oestrogen causes these mammary changes we do not know. Turner and his colleagues [Mixner & Turner, 1941] have suggested that oestrogens produce some sensitization



Table 1. *Treatment and onset of lactation in the five experimental groups*

Group	No. of sheep	Implantation of 50 mg. tablets of stilboestrol		Days between implantation and onset of lactation	
		No.	Site	Individual	Mean
1	6	3	Neck	14, 15, 33, 37, 45, 47	32
2	6	1	Udder	9, 10, 10, 10, 12, 15	11
3	3	1	Neck	18 (others did not lactate)	
4	3	2	Udder	12, 25, 42	26
5	3	3	Udder	All about 30	30

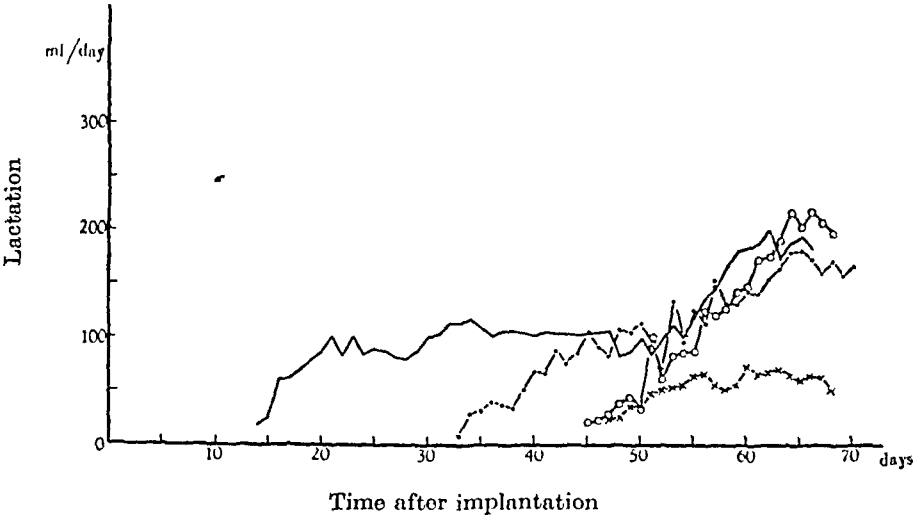


FIG. 1. Lactation curve of maiden sheep implanted with 150 mg. of diethylstilboestrol in the neck. Implantation on day 0.

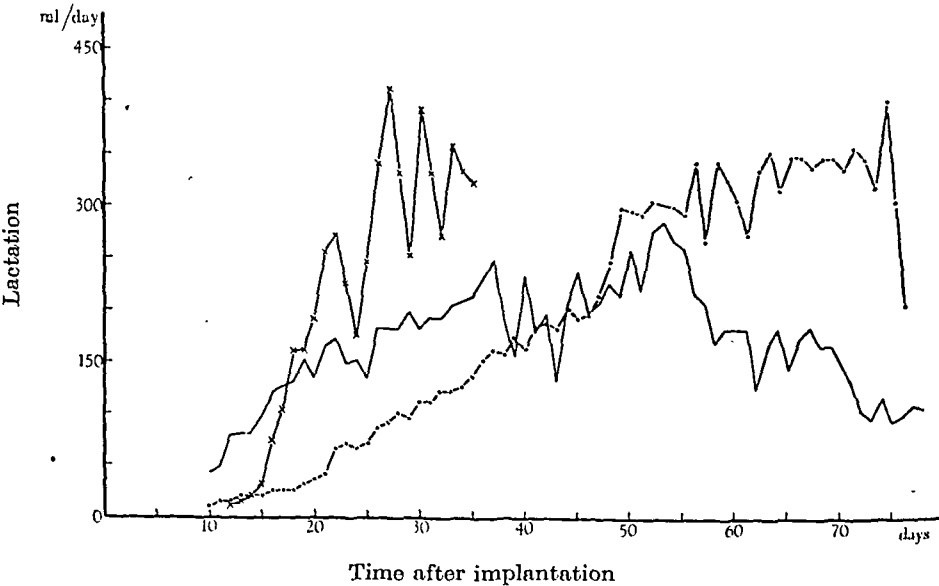


FIG. 2. Lactation curve of maiden sheep implanted with 50 mg. of diethylstilboestrol in the right half of the udder. Implantation on day 0.

of the mammary tissue to the action of mammogenic hormones, perhaps by enhancing the blood supply of the glands. Our experiment offers no information on this point, but does not exclude the possibility.

A further conclusion derived from our results is that there is an optimal quantity of oestrogen to be implanted locally, for when the dose was raised (in groups 4 and 5) the results were not so good.

The peak milk yields attained in the sheep implanted in the udder were satisfactory and were reached earlier than in the animals implanted in the neck. This aspect of the subject is being more fully investigated in heifers at the present time.

#### SUMMARY

1. Satisfactory lactation was induced in virgin sheep implanted with a 50 mg. tablet of stilboestrol in the udder. The onset of lactation occurred 9–15 days after the implantation.

2. The same dose implanted in the neck produced lactation in only one of three sheep, and when the dose was raised to 150 mg. lactation was later and irregular in onset (14–47 days after implantation) and later in attaining a peak.

3. Doses of 100 and 150 mg. implanted in the udder produced less satisfactory results.

4. Although the implantations in the udder were all made in the right half, the response was uniform in both halves.

5. It is concluded that the oestrogens have a direct action on the udder, though the possibility of pituitary hormones playing a role is not excluded.

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# HALOGEN-SUBSTITUTED OESTROGENS RELATED TO TRIPHENYLETHYLENE

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Various derivatives of triphenylchloroethylene and triphenylbromoethylene have been examined for oestrogenic activity during the past decade, notably those compounds substituted with *p*-alkoxy groups. In general, they are not very potent oestrogens, but combine long duration of action with sufficient oestrogenic activity to make them of laboratory and clinical interest. They are particularly interesting, however, in that they form a series in which halogen substitution enhances the oestrogenic activity of the molecule. Thus, triphenylchloroethylene was reported by Robson, Schönberg & Fahim [1938] to have 20 times the activity of triphenylethylene. This effect of halogen substitution is not seen in the other series of synthetic oestrogens so far studied.

A further peculiarity of triphenylchloroethylene was remarked by the present author [Emmens, 1941], who found that in contrast to triphenylethylene it has the properties of a true oestrogen, in that the median effective dose (M.E.D.) required by intravaginal application is a small fraction of that needed by subcutaneous injection. Further investigation of compounds related to triphenylethylene [Emmens, 1942] showed that this property is not shared by triphenylethylene, and that the chlorine atom cannot be effectively replaced by an ethyl group in the absence of hydroxyl groups. The converse substitution, of chlorine atoms for the ethyl groups, in 4:4'-dimethoxy- $\alpha$ : $\beta$ -diethylstilbene (diethylstilboestrol dimethyl ether), was equally unsuccessful. The last-named compound is an oestrogen of which 8  $\mu$ g. by injection, or 0.02  $\mu$ g. intravaginally, produces 50 % of positive responses in an Allen-Doisy test with mice; whereas 4:4'-dimethoxy- $\alpha$ : $\beta$ -dichlorostilbene is a pro-oestrogen, 340  $\mu$ g. of which produce 50 % of positive responses by either route. However, high oestrogenic potency is conferred on the triphenylethylene molecule by di-*p*-hydroxy substitution, and only one *p*-hydroxy group is necessary to confer true oestrogenic potency [Emmens, 1942].

The prolonged action of injected triphenylethylene or triphenylchloroethylene may be explained on the basis of their very low solubility in body fluids. A study of  $\alpha$ : $\beta$ -di-(*p*-hydroxyphenyl)- $\beta$ -phenyl-bromoethylene (D.B.E.) by Robson & Ansari [1943] indicates that, in possible addition to such an effect, the prolonged action of D.B.E., which is seen also after oral administration, is due to its storage in the body—particularly in body fat. *p*-Alkoxy-substitution of diethylstilboestrol and of oestrone also increases their duration of action after subcutaneous injection, but only slightly increases it when diethylstilboestrol dimethyl ether is given orally to mice [Emmens, 1939*b*].

As pointed out by Solmssen [1945], no comparative data have so far been published regarding the activity of *p*-hydroxy substituted compounds related to triphenyl-

chloroethylene or triphenylbromoethylene. A number of them, together with the corresponding *p*-methoxy derivatives, have now become available and are the subject of the present communication.

#### TECHNIQUE

Determinations of oestrogenic activity were made with colonies of spayed albino mice as described in previous communications [Emmens, 1939*a*, 1941]. The approximate amount of each substance required to give 50% of positive responses by systemic and intravaginal administration was calculated from results with twenty or more mice, after preliminary tests with series of graded doses on smaller numbers of animals. There would be no point in making accurate assays in the present investigation, and a determination of activity which locates the M.E.D. of each substance within 60–170% of its average value is usually sufficient. (The M.E.D. is subject to secular variation. To minimize this source of inaccuracy in determining the effects of route of administration, systemic and intravaginal tests of the same substance have been made, as far as possible, at the same time.)

Systemic administration was by two subcutaneous injections, each in 0.1 ml. of nut oil B.P. on two consecutive days. Intravaginal administration was in 50% aqueous glycerol or occasionally in distilled water, depending on the particular technique required to obtain a homogeneous suspension or solution. Previous tests [Emmens, 1941] have shown that little difference is detectable between the results with either medium. An application was made in 0.01 ml. on each of two consecutive days.

#### RESULTS

The results are shown in Table 1, which also reproduces some previous findings [Emmens, 1942] for comparison. The newly reported compounds are starred.

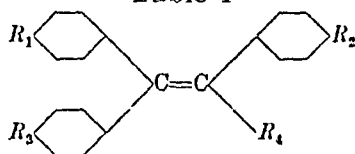
The duration of action of compounds 8, 12, 16, 17 and 18 following subcutaneous injection is prolonged. Multiples of two or three times the M.E.D. of each of these substances produce vaginal cornification which is still present in 50% of animals 2–10 weeks after injection. Compounds 8, 16 and 17 have a prolonged action even when only one M.E.D. is given. Compounds 9, 10, 13 and 14 are slightly prolonged in action; two or three times the M.E.D. produces cornification lasting for up to a week. Compounds 11 and 15 do not exhibit prolongation of action in low multiples of the M.E.D. In this series, there is thus an association between duration of action and potency; the more potent the oestrogen, the shorter its time of action. The total efficiency, if measured by the number of days during which cornification persists in a group of mice, would be less variable from substance to substance than the injected M.E.D. Following intravaginal application, no prolongation of action has been noted.

Compounds 11 and 15 are highly potent oestrogens, approaching diethylstilboestrol in potency. The M.E.D. of the latter is 0.12  $\mu$ g. by subcutaneous injection and 0.00037  $\mu$ g. when given intravaginally. It is unfortunate that a triphenylchloroethylene with phenolic substitution of two rings attached to the two different ethylene carbon atoms is not at present available, since this compound would more closely copy the architecture of diethylstilboestrol.

It will be noted that the presence of two methoxy groups in compounds 12 and 18 is associated with a much lower ratio of the median effective systemic to local dose than is seen in the corresponding dihydroxy compounds. It is not certain that

demethylation is necessary before a substance of this type can act as an oestrogen. If it is, it can take place in the vagina. Werthessen & Gargill [1945] report a personal communication from Schorr & Mazer in which the latter investigators found the excretion of diethylstilboestrol after injection of the monomethyl ether (species not disclosed), demonstrating that the conversion does in fact occur.

Table 1



Compound no.	$R_1$	$R_2$	$R_3$	$R_4$	Median effective dose in $\mu\text{g.}$ when given		
					Subcutaneously	Intravaginally	S/L ratio†
1	H	H	H	H	300	> 200	< 1.5
2	H	H	OH	H	20	44	0.45
3	OH	H	OH	H	8.0	20	0.40
4	OH	OH	H	H	15	10	1.5
5	H	H	H	$\text{C}_2\text{H}_5$	12	4.4	2.7
6	H	H	OH	$\text{C}_2\text{H}_5$	7.7	0.015	510
7	OH	OH	H	$\text{C}_2\text{H}_5$	0.90	0.00065	1400
8	H	H	H	Cl	77	0.89	86
9*	OH	H	H	Cl	{ 2.3	0.0014	1600
10*	H	H	OH	Cl		0.0013	1200
11*	OH	H	OH	Cl	0.20	0.0010	200
12*	$\text{OCH}_3$	H	$\text{OCH}_3$	Cl	40	1.2	33
13*	OH	H	H	Br	{ 2.7	0.011	250
14*	H	H	OH	Br		0.010	300
15*	OH	H	OH	Br	0.54	0.0014	390
16*	$\text{OCH}_3$	H	H	Br	{ 105	0.70	150
17*	H	H	$\text{OCH}_3$	Br		0.49	200
18*	$\text{OCH}_3$	H	$\text{OCH}_3$	Br	51	1.0	51

\* Bracketed isomers have not been separately identified.

† The ratio of the systemic to the local (intravaginal) median effective dose.

The present examples suffice to show that high oestrogenic potency is possessed by substances with no real resemblances either to the natural oestrogens or to the most potent of the other synthetic compounds so far described. The close stereochemical resemblance which hexoestrol has to the natural hormones, according to Carlisle & Crowfoot [1941], cannot be supposed to hold for compound 11, for instance. Moreover, the intravaginal activity of the monohydroxy compounds 9 and 10 is not far short of that of diethylstilboestrol, oestrone or oestradiol. (The intravaginal M.E.D. are 0.0014 and 0.0013  $\mu\text{g.}$  as against 0.00037, 0.00029 and 0.0005  $\mu\text{g.}$  respectively.) The relatively low activity and prolonged action of compounds 12, 18 and D.B.E. are paralleled by those of the alkylated diethylstilboestrols, and are not indicative of any further peculiarity of the halogen-containing series.

#### SUMMARY

1. A series of hydroxy- and methoxy-triphenyl-halogenethylenes has been examined for oestrogenic activity in ovariectomized mice. All of the compounds are oestrogens, with a higher potency when given directly into the vagina than when injected subcutaneously.

2. Monohydroxy-members of the series are potent oestrogens with subcutaneous M.E.D. of between 1.5 and 3.0  $\mu\text{g.}$ , and intravaginal M.E.D. of between 0.01 and 0.001  $\mu\text{g.}$  The dihydroxy-compounds are very potent, almost equalling the most active synthetic compounds known, and are not prolonged in action.

3. Methyl-substituted compounds resemble the corresponding diethylstilboestrol mono- or di-methyl ethers in having considerably lower potency and a prolonged action after subcutaneous injection.

Compounds 9 to 18 inclusive were generously provided by the G. W. Carnrick Co., Newark, New Jersey, U.S.A.

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# THE EFFECTS OF PREGNANCY AND RELAXIN ON THE HISTOLOGY OF THE PUBIC SYMPHYSIS IN THE MOUSE

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During pregnancy in the mouse the pubic bones become separated, and the gap is bridged by a ligament which at parturition may be 5 or 6 mm. long [Gardner, 1936]. The normal course of separation of the two bones in pregnant mice has been described in a previous paper [Hall & Newton, 1946a]. The bones begin to separate on the 13th day of pregnancy, and the gap widens an average of 1 mm. per day until parturition, which normally takes place during the night following the 19th day. After this the gap rapidly closes, is usually less than 2 mm. on the 3rd or 4th day *post partum*, but does not completely return to the virgin condition.

The changes which occur during pregnancy can be produced in the spayed mouse by the administration of an extract of pregnant rabbit serum (shown by Hisaw [1926] to contain relaxin), accompanied by a dose of oestrogen which is itself ineffective [Hall & Newton, 1946b, 1947]. The relaxin extract is ineffective if given without oestrogen.

This paper describes the histological changes which take place at the region of the symphysis pubis in the untreated pregnant mouse, and in the ovariectomized mouse treated with relaxin and oestrone. Ruth [1937] has described the histological changes which take place in the symphysis of the pregnant guinea-pig, while Gardner [1936] compared the structure of the symphysis of virgin mice with those of the multiparous animal and of mice treated for long periods with oestrogens, but did not follow the symphysis throughout the course of the first pregnancy.

## MATERIAL AND METHODS

All the mice used were sexually mature albinos, and had never previously littered. In estimating the stage of pregnancy, the midnight following the finding of the seminal plug was regarded as the end of the 1st day of pregnancy. Mice were killed each day from the 11th onwards, and also at intervals following parturition in order to study the changes that take place during regression of the ligament. The pelvis of intact and spayed virgin mice were also examined. Ovariectomized mice received daily subcutaneous injections of 25  $\mu$ g. of oestrone dissolved in ground-nut oil, alone or + 0.2 ml. of the relaxin-containing extract of pregnant rabbit serum, the injections of oestrone being started 2 days earlier than those of relaxin. The relaxin was prepared by the method of Abramowitz, Hisaw, Kleinholz, Money, Talmage & Zarrow [1942]; fuller details of experimental procedure have been given in a previous paper [Hall & Newton, 1947]. A total quantity of 1.4–1.6 ml. of relaxin extract given during 7–8 days was sufficient to produce a pubic separation of the order of that which occurs at the end of pregnancy.

The medial portions of the ischial and pubic bones with the symphyses or interpubic ligaments, were removed, fixed in 4% formol-saline, decalcified in formol-nitric, and embedded in paraffin. Serial sections 5 or 7  $\mu$  thick were cut in a transverse plane through the symphyseal region, and stained with Ehrlich's haematoxylin and eosin, or with Masson's-haematoxylin—Ponceau-fuchsin—light-green stain for connective tissue. A few sections were stained with Weigert's resorcin-fuchsin for elastic tissue.

#### OBSERVATIONS

##### *The pubic symphysis in the virgin mouse*

In the virgin mouse 3-5 months old (Pl. 1, fig. 1), the articular surfaces of the pubic bones are covered with cartilage, and meet in the medial plane to form a cartilaginous symphysis which is complete for only a very short distance. The perichondrium is continuous dorsally and ventrally over the symphysis and thickens somewhat in the neighbourhood of the symphysis. Ligaments, composed of collagen fibres and fibroblasts, connect the bones of the right and left side, anterior and posterior to the symphysis. Between the opposing articular surfaces of the pubes is a narrow cleft-like joint cavity. The perichondrium is not reflected into this cavity, which is bordered laterally by hyaline cartilage (Pl. 2, fig. 10). Anterior and posterior to the interpubic cleft, there is tissue continuity between the cartilaginous caps of the right and left pubes. The most superficial cells of the symphyseal cartilage, i.e. those nearest to the cleft, are flattened, their long axes running dorsoventrally; the deeper layers are hypertrophic. The bone in this area is cancellous and retains its primitive lamellar structure.

##### *Changes during the first pregnancy and after parturition*

The first indication of any relaxation of the pelvis appeared on the 12th day of pregnancy. Slight mobility at the symphysis could be felt on palpation, and although at dissection no interpubic gap was visible macroscopically, histological examination revealed that the articular cartilage bordering the joint cavity had proliferated so that the bones were less closely approximated. The cells in the superficial layers of this cartilage were no longer flattened and orientated parallel to the cleft, but rounded, and disposed in groups of two, four, or more, presumably owing to division of the cells; a few mitoses could be seen. There was little or no evidence of bone resorption, and the first stage in the separation of the bones is apparently brought about by proliferation of the articular hyaline cartilage.

Little change occurred during the next 24 hr., and the histological picture (Pl. 1, fig. 2) on the 13th day was similar to that seen on the previous day. The symphyseal gap was not yet visible macroscopically, nor in general accurately measurable on X-ray photographs, although in occasional mice it may measure 0.5 mm. on this day.

On the 15th day of pregnancy the interpubic gap measures about 1 mm. and thereafter the bones separate at an average rate of 1 mm. per day until parturition. In two mice examined on the 15th day the bones were connected by a ligament composed mainly of a richly cellular type of chondroid tissue, with areas of less highly organized cellular collagenous tissue, the fibres of which criss-crossed in all directions. The dorsal and ventral surfaces of the ligament were covered with a tissue similar in appearance to, and forming a plane continuous with, the periosteum.



During the last 5 days of pregnancy an intensive process of resorption of the symphysial ends of the pubic bones takes place. Osteoclasts appear in large numbers, not only at the articular tips of the bones, where the ligament is attached, but for some distance along the length of the bones. As the medial ends of the bones are thus 'eaten away', the gap widens and the ligament lengthens. On the 17th day, for instance, (Pl. 1, fig. 3; Pl. 2, fig. 11) at each end of the ligament a dense mass of spindle-shaped cells occupied the position of the most recently resorbed bone. Mitoses were very frequent amongst these cells, and between them was a network of extremely fine fibrils, many of which gave the staining reactions characteristic of collagen. It is evident that by their proliferation these cells contribute to the lengthening of the ligament: its structure now alters so that the main mass is built up of collagenous connective tissue, the fibres of which are orientated laterally (i.e. in the direction of separation) and which merge with the small masses of chondroid tissue now confined to the former articular surfaces. The cells in the tissue of the ligament are morphologically active fibroblasts. At each end of the ligament the aggregations of rapidly proliferating fibroblasts, already described, lie adjacent to the small areas of chondroid tissue, giving a picture typical of the development of fibro-cartilage (Pl. 3, figs. 12, 13); thus those fibroblasts nearest to the resorbing bone are separated by a fine fibrillar network, whilst those nearer to the chondroid tissue become rounded and some are transformed into cartilage cells. The areas of chondroid tissue merge with the looser collagenous connective tissue of the ligament. Many osteoclasts are present among the proliferating fibroblasts and at the edges of the cartilaginous tissue.

In two mice killed on the 19th and 20th days (before parturition) there was an interpubic separation of 4.5 and 5.5 mm. respectively. Except for two small areas of chondroid tissue at the former articular surfaces, the ligament consists of collagenous tissue. The intense bone resorption has resulted in the opening of some of the medullary cavities, leaving no barrier between the myeloid elements and the surrounding tissue, and near the attachments of the ligament, marrow cells can sometimes be found scattered amongst the proliferating fibroblasts. The removal of the bony plates has also freed some of the marrow sinusoids, and among the aggregations of cells at the edges of the ligament are a number of small vascular channels. Immediately after parturition the interpubic gap begins to close, and by the third or fourth day *post partum* the ligament has shrunk to about half its length (Pl. 1, fig. 5). Fresh bone is laid down, making good the previous loss; at first osteogenesis resembles that which occurs in membrane bone formation. However, there is a tendency for some of the cells in the small areas of chondroid tissue at each end of the ligament to become hypertrophic. Later, the amount of hypertrophic cartilage increases and endochondral ossification is the rule. About 1 week after parturition the symphysial plates have been regenerated, and the marrow cavities are no longer open. The disappearance of nearly all the osteoclasts immediately after parturition is quite striking.

Within 24 hr. of parturition the fibroblasts of the ligament have returned to the morphologically spindle-shaped resting condition. The ligament remains fairly vascular and its fibres are still clearly orientated for about a week. The amount of cartilage then increases at the expense of the less highly organized connective tissue, until

about 2 weeks after parturition the bones are connected by a band of avascular fibro-cartilage about 2 mm. long (Pl. 2, fig. 6). The condition then remains unchanged until the next pregnancy.

*The symphysis pubis in the spayed mouse*

After ovariectomy the symphysis pubis (Pl. 2, fig. 7) resembled that of the intact virgin female, except that very little hypertrophic cartilage was present. The articular surfaces of the bones were cartilaginous and enclosed a narrow cleft-like joint cavity. The cartilage cells were flattened and orientated parallel to the cleft.

*The effect of oestrone on the symphysis pubis of the spayed mouse*

Six mice treated with 25  $\mu$ g. of oestrone daily for 10 days were used for this investigation. In one only, an interpubic gap of 0.5 mm. was measured on the X-ray photograph; the other five showed no separation. Histologically symphyses of all six mice resembled those of the spayed controls (Pl. 2, fig. 8). No proliferation of cartilage had occurred. Therefore 250  $\mu$ g. of oestrone administered over a period of 10 days is ineffective in producing pubic separation in the spayed mouse.

*The effect of relaxin on the symphysis pubis of the spayed mouse*

Thirty spayed mice were injected with 6-8 daily doses of 0.2 ml. of the relaxin extract of pregnant rabbit serum simultaneously with oestrone (25  $\mu$ g. per day). In all the mice the pubic bones separated and a ligament was produced which in most cases measured from 3.0 to 5.4 mm. in length. Seven of these mice were used for histological investigation. During the preparation of the material a certain amount of shrinkage of the ligament took place. For example, in mouse 218 (Pl. 2, fig. 9) the width of the interpubic gap by direct measurement at autopsy was 4.0 mm., but after sectioning it measured only 3.0 mm. Similar shrinkage also occurred in the ligaments of pregnant mice—it had occurred for example in that illustrated in Pl. 1, fig. 4. Separation of the pubic bones and growth of the ligament follows the same course as during normal pregnancy, i.e. proliferation of the articular cartilage, intense bone resorption, growth in length at the edges of the ligament, and reversion of chondroid to collagenous tissue in the main mass of the ligament.

The appearance of the ligament after 8 daily doses of 0.2 ml. of relaxin extract is illustrated in Pl. 2, fig. 9 and Pl. 3, fig. 14. The articular ends of the pubic bones have been largely resorbed. At each end of the ligament the place of the most recently resorbed bone is occupied by a dense collection of cells, mainly spindle-shaped, many of them undergoing mitosis. Many capillaries thread their way between these cells, and here and there are minute spicules of bone in the last stage of resorption (Pl. 3, fig. 14).

Numbers of osteoclasts can be seen in these areas, most of them lying in close contact with the main mass of the pubic bones, or of the isolated remnants of bony spicules. As resorption of the bone progresses, the ligament grows in length by proliferation of cells at its ends. The main mass of the ligament consists of a rather loosely arranged cellular collagenous tissue, with small areas of fibro-cartilage at each end. The joint cavity is present as a very narrow cleft, its long axis parallel to the long axis of the ligament.

*The effect of pregnant mare serum on the symphysis pubis of spayed mice*

In most of the experiments the relaxing substance used was prepared from the blood of pregnant rabbits, but one small group of mice was injected with the serum from pregnant mares, or with an extract of this serum prepared by the method used for rabbit serum. The results were on the whole disappointing, in that the final pubic gap was never of the order produced by rabbit relaxin extract. However, X-ray pictures taken at intervals during the experiment were interesting. Shadows were present in the gap between the medial faces of the pubes; it was thought that these might represent isolated spicules of bone, and that an unequal process of bone resorption had taken place. Three of the mice were therefore killed and a histological examination was made of the symphyseal region of the pelvis.

Mouse 200 was injected with eleven daily doses of 0.4 ml. of concentrated serum (each dose containing 1.2 ml. of serum, dried and redissolved in distilled water) together with the usual simultaneous treatment with oestrone. On the day after the last injection the pubic gap measured 1.4 mm. on the X-ray photograph. This measurement was obtained by ignoring the shadows which were thought to be spicules of bone in the interpubic gap.

Histological examination showed that the interpubic gap was partly filled by a mass of cartilaginous tissue, bordered dorsally and ventrally by continuations of the periosteum, and enclosing a branched joint cavity (Pl. 3, fig. 15). The articular surfaces of the pubic bones were irregular in outline, and in places reduced to thin bony plates. Spicules of bone, some of them separated from the main mass, extended medially into the gap. Small isolated spicules of bone remained within the mass of interpubic cartilage; in places groups of a few hypertrophied cartilage cells were present adjacent to these spicules and to the medial faces of the pubes. Large numbers of osteoclasts could be seen in contact with the medial ends of the pubes and of the bone spicules and in the substance of the interpubic cartilage. During the eleven days of the experiment intense but unequal resorption of bone had evidently taken place. The gap so produced had been partly filled by cartilaginous tissue, but small isolated areas of incompletely resorbed bone still lay within the substance of this cartilage.

Shadows in the interpubic gap were noticed also on several occasions in X-ray photographs taken during early stages of interpubic separation, experimentally produced by pregnant rabbit serum or its relaxin-containing extract, and were thought to represent partly or completely isolated spicules of resorbing bone. Histological investigation confirmed this.

One spayed mouse treated with ten daily injections of 0.1 ml. of pregnant rabbit serum (together with the usual simultaneous treatment with oestrone) developed an interpubic separation of 1.0 mm. Microscopic examination showed that a moderate degree of bone resorption had taken place and some proliferation of cartilage had occurred to fill the gap, but nothing approaching the rapid proliferation produced by the relaxin concentrate.

## DISCUSSION

The resorption of the pubic bones which occurs in intact mice during normal pregnancy and in spayed mice treated with relaxin is both intense and striking, and certainly contributes to the widening of the birth canal. But it is not the only, nor probably the most important, factor. Examination of the dissected pelvic girdle and

of X-ray photographs shows that the entire pubic and ischial bones of each side have moved away from the midline. Resorption, by thinning the bones, especially at the symphyseal tip, merely contributes to the widening of the gap.

The resorption of the bone tips, together with the laying down of clearly orientated collagen bundles seems to suggest a tissue response to a force imposed in some way from without, rather than a definite chemical action on the cells themselves. The first histological change is always proliferation of the articular hyaline cartilage. Osteoclasts in significant numbers appear a little later and bone resorption becomes more intense as pregnancy advances, and is paralleled by very rapid proliferation at the ends of the ligament. Whether the bones are forced apart in response to pressure from the rapidly lengthening ligament or whether some force operates on the bones directly, the tissue proliferation to fill the gap being a secondary rather than a contributory factor, is not clear.

Ruth [1937] described a rather intense process of resorption of the pubic and ischial bones of the guinea-pig during pregnancy, but found no evidence of a significant increase in the number of osteoclasts. He considered that the comparative absence of these cells provided substantial evidence that they have no direct function in bone dissolution. In the present series of sections, both of pregnant mice and of those treated with relaxin, the increase in the numbers of osteoclasts in the areas of most intense resorption is so striking, and so many of them are to be found in intimate connexion with pieces of necrotic bone, that it is difficult to believe they play no part in the resorptive process.

In the same paper Ruth describes very intense vascularization of the ligament during the later stages of pregnancy in the guinea-pig. Some capillaries and blood sinusoids were present in the fully developed ligament of the mouse, especially near each end, but the vascularization was not so extensive as that pictured by Ruth. Many of the capillaries and blood sinusoids present among the proliferating fibroblasts were probably blood channels liberated from the marrow cavities by the dissolution of the bony plates.

The ligament at first consists of a band of cartilage covered dorsally and ventrally by extensions of the periosteum. As pregnancy advances, and the ligament becomes longer, most of the cartilage reverts to a more primitive type of collagenous tissue, except at the extreme ends where small areas of chondroid tissue remain. These islands of cartilage are in close proximity to the sites of most active proliferation. It is evident that the aggregations of proliferating fibroblasts near the symphyseal tips produce by their activity chondroid tissue which adds to the length of the ligament. But these areas of active proliferation occupy the positions of the most recently resorbed bone, and amongst the fibroblasts and in intimate contact with the periphery of the cartilage there remain considerable numbers of osteoclasts (Pl. 3, fig. 13) and it seems possible that these latter may be agents in the reversion of the cartilage to a looser type of connective tissue, perhaps assisting in some way in the removal of the binding ground substance. The cartilage cells, thus freed from pressure, may once more change over into fibroblasts.

Gardner [1936] produced long interpubic ligaments (4-5 mm. in length) and resorption of the medial portions of the pubic bones in female mice by administering various oestrogenic hormones. In some of our earlier experiments, by continuing the

injections of oestrone for a period of 3 weeks we were able to produce in a few mice a pubic separation up to 1.7 mm. Unfortunately no histological examination was made of these mice, but it was quite clear from the X-ray photographs that changes on the scale of those described by Gardner had not occurred. It is probably significant that Gardner began treatment at a much earlier age, and that his mice retained their ovaries.

#### SUMMARY

1. A description is given of the histological changes which occur at the symphysis pubis of the mouse during the first pregnancy, and of the spayed mouse under the influence of relaxin.

2. During the last six days of pregnancy the pubic bones move apart and a ligament 4–6 mm. long occupies the interpubic gap. The histological changes involved consist of proliferation of the articular hyaline cartilage; rather intense resorption of the medial ends of the pubes, especially at the symphyseal tips; lengthening of the ligament by formation of new cartilage from fibroblasts at each end; and reversion of this cartilage to a more primitive type of clearly orientated collagenous connective tissue.

3. The gap begins to close immediately after parturition and 4 days later the ligament has usually shrunk to half its length. Rapid bone repair results in regeneration of the symphyseal plates in about 1 week. The fibroblasts of the ligament return to the resting condition within 24 hr. *post partum*, and the connective tissue is replaced by cartilage. Two weeks after parturition the bones are connected by a cartilaginous band about 1 mm. long, and the condition then remains unchanged until the next pregnancy.

4. Oestrone, in doses of 25  $\mu$ g. per day for 10 days, produced no change at the symphysis pubis of the spayed mouse.

5. Relaxin, given in combination with ten daily doses of 25  $\mu$ g. of oestrone produced pubic separation and ligament formation similar to that which occurs at the end of pregnancy. The histological changes involved and the structure of the ligament were identical with those in normal pregnancy.

6. The possible factors involved in the separation are discussed. Bone resorption contributes to the widening of the gap, but is not the primary cause.

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## EXPLANATION OF PLATES

## Plate 1

FIG. 1. Transverse section through the symphysis pubis of a virgin mouse  $4\frac{1}{2}$  months old. The articular surfaces of the pubes are cartilaginous and enclose a cleft-like joint cavity. The periosteum is continued over the symphysis.  $\times 32$ .

FIG. 2. Transverse section through the symphysis pubis of a mouse killed on the 13th day of the first pregnancy. The articular cartilage bordering the joint cavity has proliferated so that the bones are less closely approximated.  $\times 32$ .

FIG. 3. Transverse section through the symphysis pubis and ligament of a mouse killed on the 17th day of the first pregnancy. Considerable resorption of bone has taken place at the symphyseal ends of the pubes, and the interpubic gap is filled by a ligament 2.1 mm. long, composed partly of cartilage and partly of cellular collagenous tissue, the fibres of which are not very well orientated.  $\times 32$ .

FIG. 4. Transverse section through the pubic symphysis and ligament of a mouse killed on the 20th day of the first pregnancy (before parturition). Further resorption of bone has taken place, and the interpubic gap measures 5.5 mm. The main mass of the ligament consists of cellular collagenous tissue (the fibres being orientated in the direction of separation) with small areas of fibro-cartilage at the ends.  $\times 32$ .

FIG. 5. Transverse section through the symphysis pubis and ligament of a mouse killed during the 4th day after parturition. The ligament measures 1.9 mm.  $\times 32$ .

## Plate 2

FIG. 6. Transverse section through the symphysis pubis of a mouse killed on the 23rd day after parturition. Active osteogenesis is in progress but the symphysis has not returned to the virgin condition. An interpubic gap measuring 0.9 mm. is present, bridged by fibro-cartilage over which the perichondrium is continuous dorsally and ventrally.  $\times 32$ .

FIG. 7. Transverse section through the symphysis pubis of an untreated spayed mouse,  $4\frac{1}{2}$  months old, killed 32 days after removal of the ovaries. Similar to Pl. 1, fig. 1, except that no hypertrophic cartilage is present on the articular surfaces.  $\times 32$ .

FIG. 8. Transverse section through the symphysis pubis of a spayed mouse treated with ten daily injections of  $25\mu\text{g.}$  of oestrone. The histological picture is similar to that of the control (Fig. 7), except that a little hypertrophic cartilage is present at the symphyseal ends of the pubes.  $\times 32$ .

FIG. 9. Transverse section through the symphysis pubis of a spayed mouse treated with eight simultaneous daily injections of  $25\mu\text{g.}$  of oestrone and 0.2 ml. of relaxin extract, after two priming doses of  $25\mu\text{g.}$  of oestrone. Bone resorption and cellular proliferation has produced a pubic separation and ligament 4.0 mm. long, identical in appearance with that in Pl. 1, fig. 4.  $\times 32$ .

FIG. 10. High-power photograph of part of the articular surfaces of the pubic bones of a virgin mouse (that shown in Pl. 1, fig. 1). The cartilaginous tips of the two bones are separated by a narrow joint cleft.  $\times 340$ .

FIG. 11. Small area of bone and adjacent articular cartilage seen on the left in Pl. 1, fig. 3 (17th day of pregnancy). Bone resorption is resulting in the opening of some of the marrow cavities (e.g. immediately above the megakaryocyte which can be seen left centre). The mass of proliferating fibroblasts in the lower half of the field is contributing to the growth of the ligament.  $\times 340$ .

## Plato 3

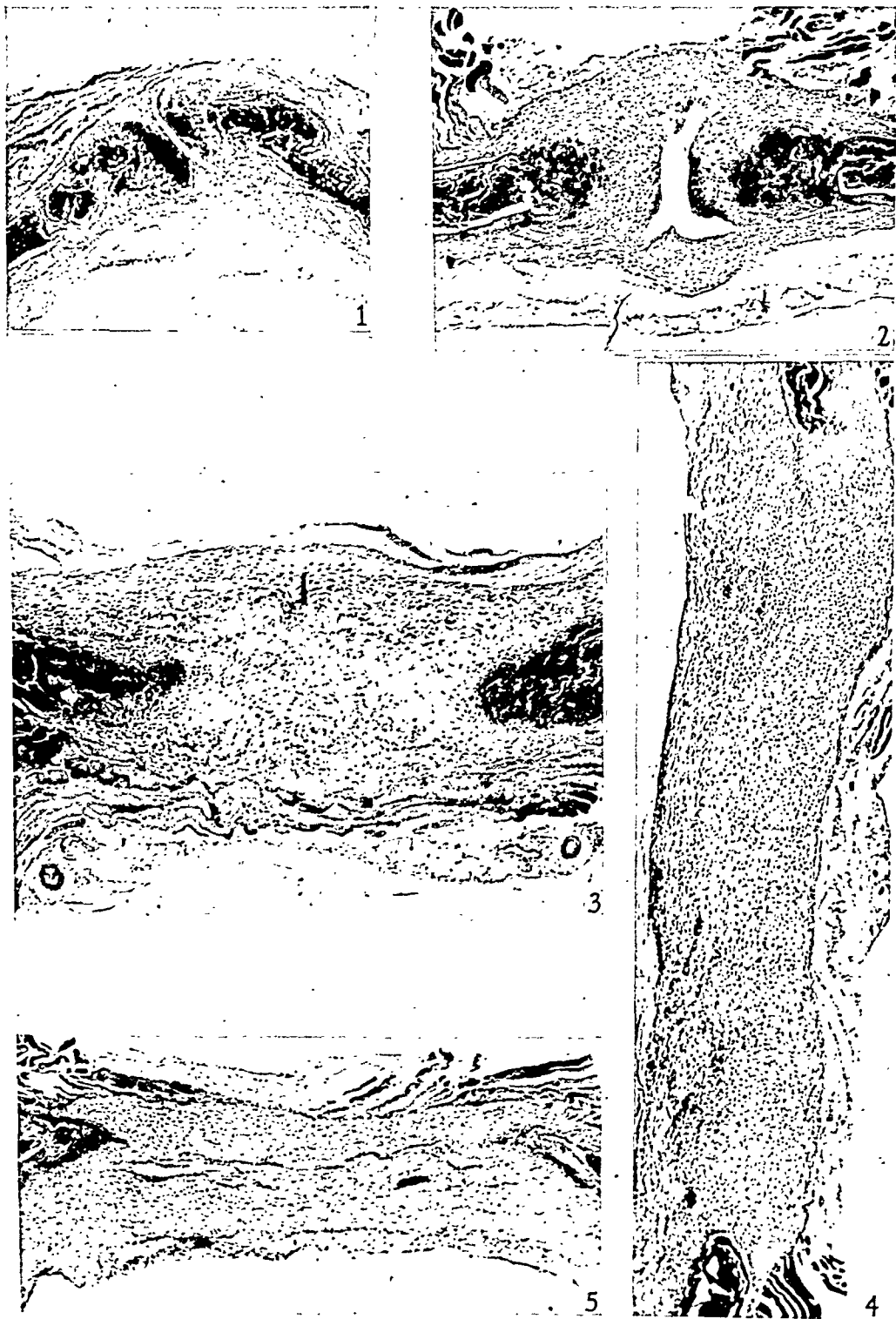
FIG. 12. Small area of 'articular' surface of bone and end of ligament of a pregnant mouse killed on the 19th day of pregnancy. Intense bone resorption has reduced the symphyseal surface of the pubis to a plate of bone. Many osteoclasts are present. The numerous proliferating fibroblasts are contributing to the length of the ligament.  $\times 340$ .

FIG. 13. Small area of resorbing pubic bone and end of ligament of a pregnant mouse killed on the 19th day of pregnancy, showing the development of chondroid tissue from proliferating fibroblasts at the end of the ligament. Two osteoclasts can be seen (right lower centre) in intimate contact with the cartilage matrix.  $\times 340$ .

FIG. 14. Spayed mouse given the same treatment as that in Pl. 2, fig. 9. Photograph shows end of ligament with tip of resorbing pubic bone in top left-hand corner. Dense aggregations of proliferating cells occupy the position of the recently resorbed bone; a few osteoclasts are present amongst these cells. Small isolated remnants of necrotic bone in the last stages of resorption can be seen amongst the proliferating cells (lower centre of photograph).  $\times 340$ .

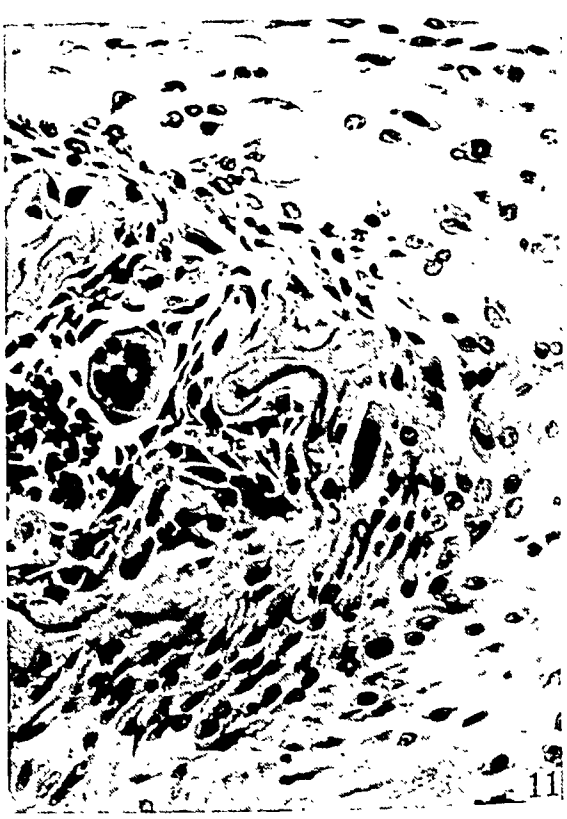
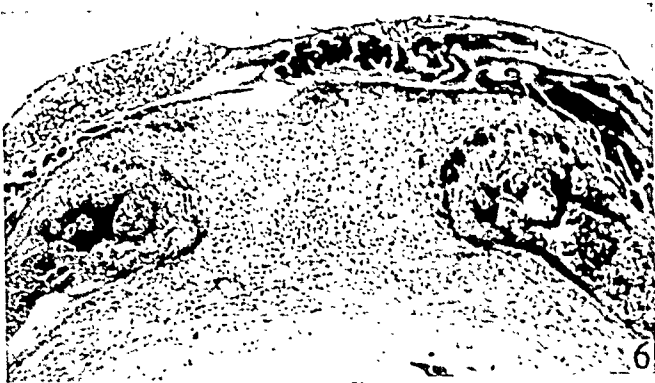
FIG. 15. Spayed mouse treated with twelve daily injections of 1.2 ml. of pregnant mare serum simultaneously with  $25\mu\text{g}$ . per day of oestrone after two priming doses of oestrone. Interpubic gap 1.4 mm. Intense resorption has reduced the tip of the pubic bone (seen on right) to a bony plate. Half of the cartilaginous interpubic band is shown with the joint cleft. A small mass of hypertrophic cartilage is present near the centre of the interpubic cartilage. Two small isolated areas of necrotic bone can be seen to the right of the cleft, inside the perichondrium in the lower half of the photograph.  $\times 115$ .

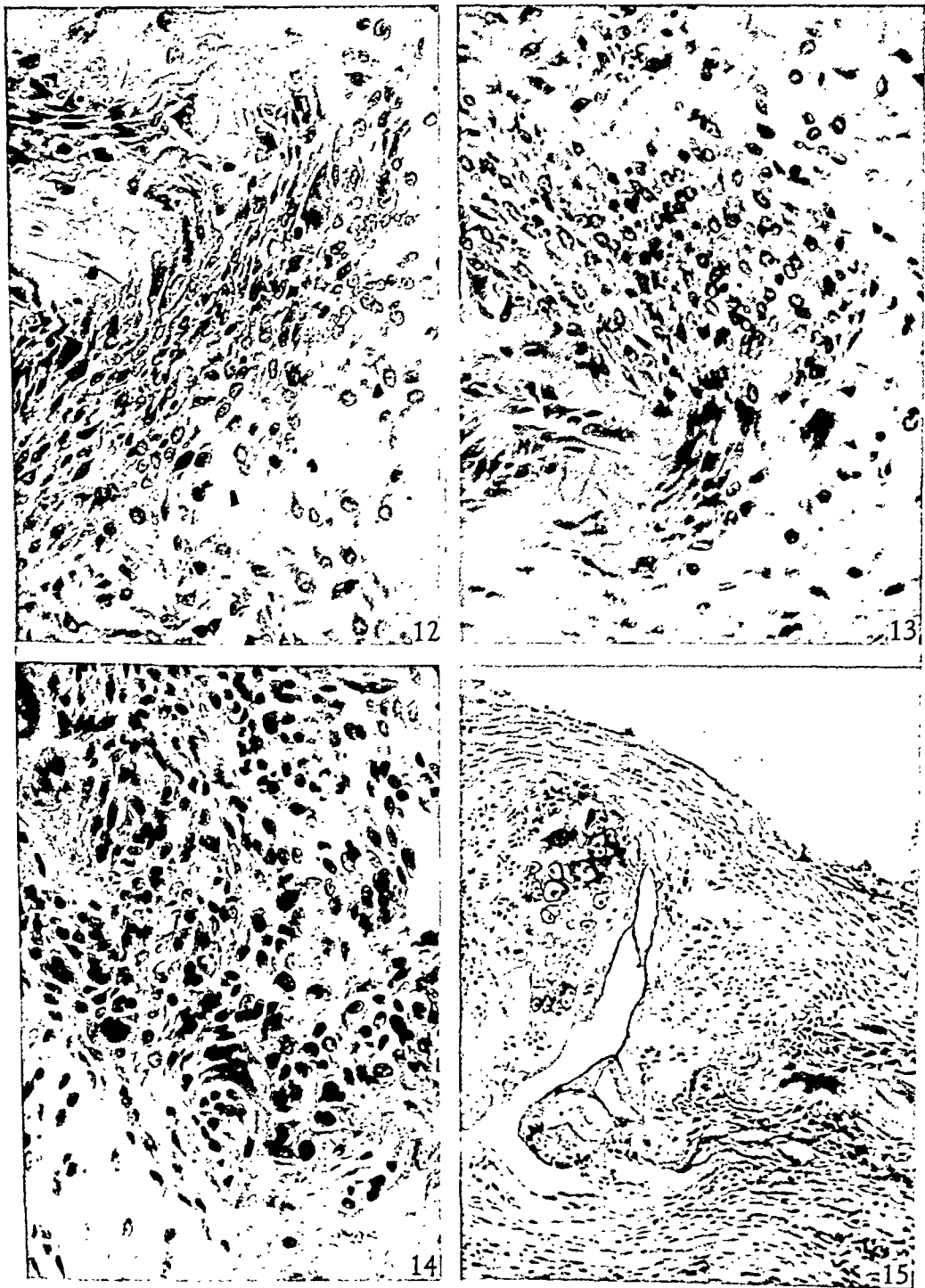
Figs. 1-9 were prepared by Mr F. A. Murray; Figs. 10-15 by Mr F. Beckwith.



Figs. 1-5







FIGS. 12-15



# THE HORMONAL PROPERTIES OF PERHYDRODIETHYLSTILBOESTROL

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(Received 20 May 1947)

That the perhydro-derivative of diethylstilboestrol should be androgenic just as diethylstilboestrol is oestrogenic was predicted by Schueler [1946]. This prediction was based on the hypothesis that a compound may possess androgenic activity if the active, polar groups are at a distance of 9–10 Å. and are, in comparison with the active groups found in oestrogenic compounds, of somewhat weaker hydrogen bond-forming character (e.g. secondary alcohol hydroxyl groups).

Many syntheses of perhydro-derivatives of diethylstilboestrol have been described [Lane & Wallis, 1943; Major, Christman & Folkers, 1944; Hoehn & Ungnade, 1945; Ungnade & Ludutsky, 1945] and it has been shown that six isomers exist [Ungnade & Ludutsky, 1945], but unfortunately the hormonal activities of the products were not reported. The corresponding partly re-oxidized product, the ketone, diethyl(*p*-cyclohexenonyl)(*p*-cyclohexanoly)ethane, is claimed to have an androgenic activity corresponding to '1 capon unit per mg.' [Schering Corporation, 1946].

There were available to us, by the courtesy of our colleague Dr W. M. Duffin, two preparations of perhydrodiethylstilboestrol: first, a crude oily product which is expected to contain several isomers; secondly, a crystalline isomer of m.p. 188–188.5°. Both have been tested for oestrogenic and androgenic activity and the crude oily product has also been tested for corticosterone-like and progesterone-like activity.

## EXPERIMENTAL

### *Oestrogenic properties*

The oestrogenic activity of each preparation was compared with the activity of diethylstilboestrol, both being injected subcutaneously in arachis oil into groups of spayed immature rats and the uteri weighed [Bülbring & Burn, 1935]. The activities of the perhydro-derivatives, expressed as ratios of the activity of diethylstilboestrol, together with the calculated limits of error corresponding to  $P = 0.95$  are shown in Table 1. The results are derived from tests in which litter-mate groups containing six inbred Wistar rats were used.

Table 1. *The relative oestrogenic activities of crude oily perhydrodiethylstilboestrol, crystalline perhydrodiethylstilboestrol, and of diethylstilboestrol.*

Substance	Relative activity	Limits of error ( $P = 0.95$ )
Diethylstilboestrol	1	—
Crude, oily perhydrodiethylstilboestrol	0.063	0.038–0.104
Crystalline perhydrodiethylstilboestrol (m.p. 188–188.5°)	0.0017	0.0008–0.004

*Androgenic properties*

The effects of the perhydro-derivatives and of testosterone propionate on the capon's comb were compared. Dissolved in arachis oil, the compounds were injected daily for 6 days into the pectoral muscles, and the sum of the length and height of the comb recorded daily. Whereas no increase in the size of the comb followed the injection of a total dose of 1, 10 or 100 mg. of the crude perhydrodiethylstilboestrol or of 6 mg. of the crystalline isomer, a total dose of 0.1 mg. of testosterone propionate caused an increase of 5.3 mm. (standard error = 0.3). Since insufficient material was available it was not possible to administer larger doses of the crystalline preparation. On this account use was made of a more sensitive method in which the drugs are injected into the comb itself. By this route no increase in the size of the comb followed the injection of 6 mg. of perhydrodiethylstilboestrol, m.p. 188–188.5°, in two birds, whereas 0.01 mg. of testosterone dipropionate resulted in increases of 3 and 5 mm. in the same birds.

The effect of the perhydro-derivatives on the weight of the prostate and seminal vesicles of the spayed rat was also determined. Castrated when three or four weeks old, the animals were left for two weeks before being used in the tests. The drugs were injected subcutaneously into litter-mate groups of rats on each of five successive days, in a total volume of 1.0 ml. of arachis oil. The mean combined weights of the prostate and seminal vesicles on the seventh day of each of three tests are shown in Table 2.

Table 2. *The mean weights of prostates and seminal vesicles, in litter-mate groups of immature spayed rats following treatment with perhydrodiethylstilboestrol or diethylstilboestrol*

Total doses of compounds	No. of rats	Combined weight of prostate and seminal vesicles (mg. $\pm$ S.E.)
<i>Test 1:</i>		
Crude perhydrodiethyl-		
stilboestrol: 12.5 mg.	6	53.7 $\pm$ 2.9
25.0 mg.	6	51.1 $\pm$ 2.3
50.0 mg.	6	57.4 $\pm$ 3.7
<i>Test 2:</i>		
Crystalline perhydrodiethyl-		
stilboestrol: 40.0 mg.	4	41.8 $\pm$ 1.0
Arachis oil (Controls)	4	23.3 $\pm$ 2.0
<i>Test 3:</i>		
Diethylstilboestrol: 0.1 mg.	4	41.8 $\pm$ 2.8
1.0 mg.	4	53.3 $\pm$ 4.0
Arachis oil (Controls)	4	19.5 $\pm$ 2.3

The increase in the weight of the male organs of the castrated rat following treatment with the perhydrodiethylstilboestrols is not significantly different from that due to doses of diethylstilboestrol of equivalent oestrogenic potency. The effect of oestrogen in this respect is due to hypertrophy of smooth muscle in the prostate and seminal vesicles [Freud, 1933].

It is concluded from these tests that the androgenic activity, if any, possessed by these perhydro-derivatives is at least a thousand-fold less than the activity of testosterone propionate, and therefore is equivalent to less than 0.05 of an international androgenic unit per mg.

*Progesterone-like and corticosterone-like properties*

No progestational proliferation of the immature rabbit uterus [McPhail, 1934] followed the subcutaneous injection of total doses of 50 or 100 mg. of the crude perhydrodiethylstilboestrol. These same doses did not prolong the survival of adrenalectomized drakes.

## DISCUSSION

An examination of two preparations of perhydrodiethylstilboestrol, one a crude oily product expected to contain a mixture of the isomers and the other a crystalline isomer melting at 188–188.5° C., has shown these derivatives to be oestrogens of much lower potency than diethylstilboestrol. The crude oily product possesses about 6% and perhydrodiethylstilboestrol (m.p. 188–188.5° C.) about 0.02% of the activity of diethylstilboestrol. The relatively high potency of the crude product may possibly be due to contamination with unreduced diethylstilboestrol. It has been shown that the isomer melting at 188–188.5° C. does not inhibit lactation in the rat [Brownlee & Green, 1947].

Since neither of these products exhibited androgenic activity, the prediction that perhydrodiethylstilboestrol should be androgenic [Schueler, 1946] has not been realized. On the other hand the partially re-oxidized product, diethyl(*p*-cyclohexenonyl)(*p*-cyclohexanoly)ethane, has been claimed to possess androgenic activity equivalent to '1 capon unit per mg.' [Schering Corporation, 1946].

## SUMMARY

1. Two preparations of perhydrodiethylstilboestrol, one a crude oily product which is expected to contain several isomers and the other the isomer, m.p. 188–188.5° C., have been examined for hormonal activity.

2. Both the crude oily product and the crystalline isomer possess oestrogenic activity, having respectively about 6% and 0.2% of the activity of diethylstilboestrol.

3. No androgenic activity could be demonstrated in either of the preparations of perhydrodiethylstilboestrol, nor could progesterone-like nor corticosterone-like activity be demonstrated in the crude oily perhydrodiethylstilboestrol.

Our thanks are due to Dr W. M. Duffin of the Wellcome Chemical Research Laboratories for the supplies of the perhydrodiethylstilboestrol.

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# ASSAY OF THYROIDAL ACTIVITY BY A CLOSED VESSEL TECHNIQUE

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(Received 17 June 1947)

The work of Harington & Randall [1929] provided a convenient chemical method, based on the estimation of acid-insoluble iodine, for the assay of dried thyroid preparations. This method appears to be satisfactory for thyroid preparations, in which all the acid-insoluble iodine is thyroxine iodine. As a result, the biological assay of thyroidal activity has been much neglected and has mainly been used for determining the activity of substances related to thyroxine. In the last few years, artificially iodinated proteins (mostly iodinated casein), which possess high thyroidal activity, have come into use. These iodinated proteins contain a large amount of acid-insoluble iodine, and it is evident that only a small and variable proportion of it represents thyroxine or other substance with similar activity [Deanesly & Parkes, 1945*b*]. Chemical assay of such preparations is thus of little value, and the whole problem of the biological assay of thyroidal activity has had to be revived.

Several different types of test on mammals have been used from time to time, the most important being those depending on changes in respiratory metabolism. With this type of test it is usual to maintain mice, guinea-pigs, or rats, under conditions as near basal as possible, and to determine either the oxygen intake [Gaddum, 1930] or the carbon dioxide output [Mørch, 1929]. These methods are extremely cumbersome and time-consuming if a reasonable degree of accuracy is to be achieved on a large number of preparations. Other tests, such as those based on resistance to acetonitrile and on modification of the growth curve of guinea-pigs, appear to be unsatisfactory.

Methods more promising for large-scale work have been evolved on tadpoles, in which thyroid preparations rapidly cause premature metamorphic changes. For a long time the chief difficulty about the tadpole test was the provision of regular supplies of tadpoles, but this difficulty can now be overcome by the use of *Rana pipiens* [Reineke & Turner, 1942], or much more easily by the use of *Xenopus laevis* [Deanesly & Parkes, 1945*a*], which can be made to breed in the laboratory all through the year. However, the fact that di-iodo-tyrosine is active in the tadpole test, and has not so far been found to be active in mammals, raises some slight doubt as to the specificity of the tadpole test.

Experiments had been carried out in another connexion on the survival time of tadpoles in sealed vessels containing no air space [Deanesly & Parkes, 1945*a*], and we considered whether the use of some criterion of this kind would enable tadpoles to be used for a metabolic test. It seemed, however, that if survival time with a limited oxygen supply was to be used as a criterion of metabolic rate, then a small mammal such as the mouse, kept in a sealed vessel, might provide a more convenient and much more acceptable test object than the tadpole. We decided, therefore, to investigate the survival time of mice in sealed vessels, with and without preliminary treatment

with dried thyroid preparations. In reviewing the literature relevant to this investigation it appeared that the survival time of mammals in sealed vessels had been studied as long ago as 1878 by Paul Bert. His experiments, as tabulated in Hitchcock's translation [1943] of *La Pression Barométrique*, were carried out on cats, dogs, rabbits and guinea-pigs, confined in bell-jars of such a size as to contain enough air to support life for 1–2 hr. at ordinary pressure (roughly 6–7 l. volume per kg. animal weight). Terminal carbon dioxide was highest (16.4%) and oxygen lowest (2.3%) in the case of the guinea-pig. Bert's experiments in this particular field were not apparently extensive enough to give quantitative results.

By contrast with this early work, nearly all the modern work on the effects of anoxia has been carried out with oxygen tensions artificially reduced and regulated. Resistance to anoxia so produced is increased by thyroidectomy [Streuhli, 1918; Duran, 1920; Houssay & Rietti, 1932; Barach, Eckman & Molomut, 1941; Leblond, 1944; and others]; in contrast, resistance is decreased by administration of thyroid or thyroxine [Duran, 1920; Rydin, 1928; Campbell, 1938; Leblond, 1944]. Duran realized that the reaction could be used as the basis of a method for assaying thyroid. However, the fact that heavy apparatus is required to reduce and control oxygen tension artificially seemed to justify an attempt to evolve a technique based merely on the survival time of animals incarcerated in closed vessels at ordinary pressure. It was anticipated that, under these conditions, the survival time of mice treated with thyroid would be decreased not only by decreased resistance to anoxia but also by increased oxygen consumption.

#### MATERIALS AND TECHNIQUE

##### *Animals*

The animals used were young albino mice of the Medical Research Council's colonies maintained at Mill Hill. They were fed on the cubes described by Parkes [1946*a*] and given water from a bottle. Food was left with them until immediately before they were used in a test. The conditions were not in other respects those of a basal metabolism experiment, and the withholding of food was thought to be inadvisable because of its known effects on resistance to anoxia. Except where otherwise stated the mice weighed 18–22 g. at the beginning of the experiment.

##### *Vessels*

The vessels chosen were glass Kilner jars of the type ordinarily used for fruit bottling. Three standard sizes were used: 1, 2 and 3 lb. The individual specimens of any one size varied little in volume. Fifty 1 lb. jars ranged in capacity from 470 to 480 ml.; one hundred 2 lb. jars from 860 to 880 ml.; one dozen 3 lb. jars from 1100 to 1120 ml. The glass lid of the Kilner jar is fitted with a rubber washer and secured by a metal screw-band giving an effective airtight seal.

A small quantity of sawdust was placed in the jars before use to collect the urine and faeces and to facilitate cleaning. When soda lime or calcium chloride was used as an absorbent it was enclosed in a perforated zinc cylinder (5 × 1 in.).



*Temperature regulation*

Except where otherwise stated the experiments were carried out on open shelves in a heated room, in which the temperature was maintained and controlled between 22.5 and 23.5° C.

*Gas analysis*

When samples of the enclosed air were required for analysis the glass lids of the jars were replaced by bakelite ones fitted and secured in the same way. These lids had two circular apertures  $\frac{1}{2}$  in. in diameter, one holding a rubber sampling bung, and the other, when in use, fitted with a thermometer or a manometer connexion.

Air samples were taken into the Brodie sampling apparatus, and analysed with the Haldane gas-analysis apparatus.

*Substances administered*

(1) The commercial thyroid preparation used was Batch 628,423, tadpole results with which have already been described [Parkes, 1946*b*]. For injection, this material was weighed out, ground down in a mortar with 0.9% saline, and diluted to form a suspension containing 500 mg./100 ml. of saline.

(2) A partially purified thyroid preparation, Ox Thyroid II, and iodinated casein CB 3/62, both previously described [Parkes, 1946*b*; Deanesly & Parkes, 1945*b*], were also used. These, together with the control casein, were prepared for injection as before [Parkes, 1947].

All the preparations were given by subcutaneous injection of 1 ml. of solution or suspension.

*Experimental procedure*

The mice were weighed immediately before test and, except where otherwise stated, were distributed singly in 2 lb. jars, which were laid on their sides to facilitate observation. The experiment was deemed to start when the lid was screwed down. The usual dosage group was ten animals. Survival time (s.t.) was recorded for each individual, and the mean survival time (M.S.T.) for the group obtained. After being enclosed, the mice tended to be highly active for a short preliminary period of 10–20 min. Thereafter they became quiet and often appeared to sleep for the greater part of the experiment. A progressive increase in the rate and depth of respiration was noticeable. The terminal symptoms were restlessness, lasting 5–10 min., coma and brief convulsions. The time of the last breath was taken as the end-point of the experiment.

Where the carbon dioxide in the jars was absorbed, the mice tended to be almost motionless after the first half-hour, and the respiratory movements were so slight as to be difficult to see. The terminal phase of restlessness and often the convulsions were suppressed.

## SURVIVAL TIME OF NORMAL MICE

*Size of vessel*

The s.t. of normal female mice in 1, 2 and 3 lb. jars was compared. The results of two experiments, carried out on different days, are shown in Fig. 1.

The M.S.T. was prolonged by increasing the jar size, as expected, but it was prolonged out of proportion to the increase in jar volume. Thus, the M.S.T. was doubled by

increasing the jar volume from 475 to 872 ml., a 1.8-fold increase in volume, and trebled when the volume was increased from 475 to 1102 ml., a 2.3-fold increase in volume. The s.t. in 475 ml. of air (25 l./kg. body weight) was similar to that recorded by Bert for a guinea-pig confined in about 7 l. of air per kg. body weight, so that, on the basis of Bert's one experiment, the air volume/body weight ratio to give equal survival time seems to be different for the two species.

In the 3 lb. jars the range of individual s.t. in the group of ten mice was increased to over 2 hr. (one mouse survived for  $5\frac{1}{2}$  hr.), and the experiment became inconveniently long. It was decided to discard the 3 lb. jars and to investigate other variables by the use of the 1 or 2 lb. sizes.

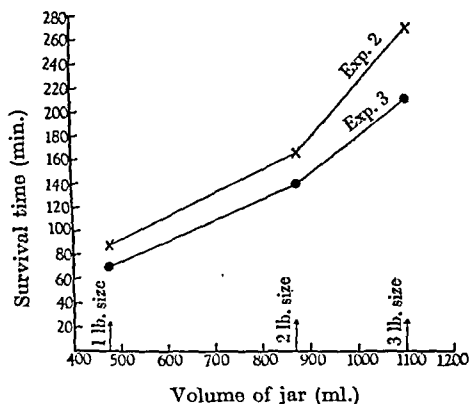


FIG. 1.

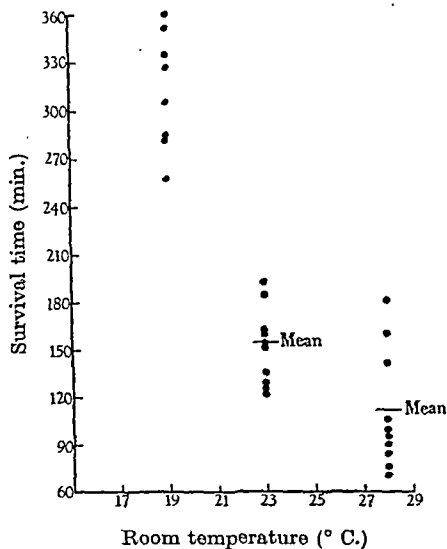


FIG. 2.

FIG. 1. Effect of jar volume on survival time of normal mice. Each point shows the average for 10 mice.

FIG. 2. Effect of room temperature on survival time of normal mice. Each point is for an individual mouse. Two mice in the group at 19° C. survived more than 390 min.

### Temperature

The effect of environmental temperature on s.t. was studied on three groups of ten normal female mice. Ten jars were maintained at 19° C. (unheated room), ten at 23° C. (thermostated room), and ten at 28° C. (incubator). The results are shown in Fig. 2 and indicate that altering the temperature had a distinct effect. The s.t. was greatly prolonged at the lowest temperature; two of the mice were still alive after 390 min., when the experiment was concluded.

From a practical point of view, an environmental temperature of 23° C. presented several advantages. The s.t. was of convenient length and varied least at that temperature. Moreover, it is much simpler to perform the experiment in a temperature-controlled room rather than to use an incubator, and 23° C. is an easy temperature to maintain and a comfortable one for the operator.

The relation between the temperature inside the closed vessel and the temperature of the surrounding atmosphere was studied, using five normal female mice weighing

17–19 g., enclosed in jars fitted with bakelite lids carrying thermometers. The room temperature, the temperature inside an empty jar, and inside each of the five jars containing a mouse, was noted at half-hour intervals. The results showed that, within a few minutes, the temperature inside the jars became 1.0–2.0° C. higher than the external temperature and thereafter remained steady. There was little variation from jar to jar during the course of the experiment. In subsequent experiments internal temperatures were not usually recorded.

The metabolic rate of mice is basal at 28° C. and is raised progressively as the temperature is reduced below that level [Mørch, 1929]. If the s.t. of mice in closed vessels depended entirely upon the rate of oxygen consumption it should be maximal at 28° C., and should be decreased by lowering the temperature. The results obtained suggest that some factor other than, or in addition to, the metabolic rate determines the s.t. of mice in closed vessels at different temperatures.

### *Seasonal variation*

The m.s.t. of groups of ten normal mice has shown no seasonal variation in experiments carried out from November to July, though at 23° C. it has varied from 109 min. to 150 min. on different days in the same month (Table 1), and time-to-time variation in response within months is shown by analysis of variance to be highly significant.

Table 1. *Mean survival time of normal male mice according to season*

Month	No. of experiments	Mean survival time (min.)	
		Range	Average
January 1947	5	109–141	120
February 1947	2	117–138	128
March 1947	17	109–148	123
April 1947	12	109–150	129
May 1947	2	122–130	126
May 1946	1	125	125
June 1946	1	123	123
December 1946	1	127	127

### *Changes in the enclosed air*

*Pressure changes.* We investigated the pressure changes inside the jars during a number of experiments and found that a slight negative pressure, usually –2 to –15 mm. mercury, was set up. A negative pressure as high as –20 mm. mercury was rarely reached. No relation between the degree of negative pressure and the s.t. was noted. As the changes were small compared to atmospheric pressure it seemed permissible to ignore them. Instead of gaseous tensions, the percentage composition of the air inside the jars during the experiment and at the time of death, under different conditions, has been determined.

*Composition of air at death in vessels of different size.* The composition of the enclosed air at the time of death of normal male mice in 2 or 3 lb. jars was determined, using five jars of each size. The result is given in Table 2. Individual variation in composition of the air showed no relation with individual variation in s.t. The m.s.t. was prolonged 50 % in the 3 lb. jars, the composition of the air at death was almost the same as in the 2 lb. jars, the mean oxygen content being about 5 % and the mean

carbon dioxide content a little over 13% for both groups. These figures may be taken to indicate the point at which constantly rebreathed air becomes lethal to mice. They indicate a higher oxygen and a lower carbon dioxide content than Bert found to be lethal to a guinea-pig.

Table 2. *The composition of the enclosed air at the time of death of normal male mice in 2 and 3 lb. jars*

	Survival time (min.)	CO <sub>2</sub> percentage	O <sub>2</sub> percentage
2 lb. jars			
Jar 1	130	13.17	4.88
2	135	13.51	5.20
3	172	13.39	4.39
4	185	14.77	4.61
5	220	13.14	5.19
Average	168	13.6	4.9
3 lb. jars			
Jar 1	170	14.29	4.51
2	175	12.83	4.15
3	235	11.69	6.89
4	305	13.90	3.73
5	335	13.37	5.27
Average	244	13.2	4.9

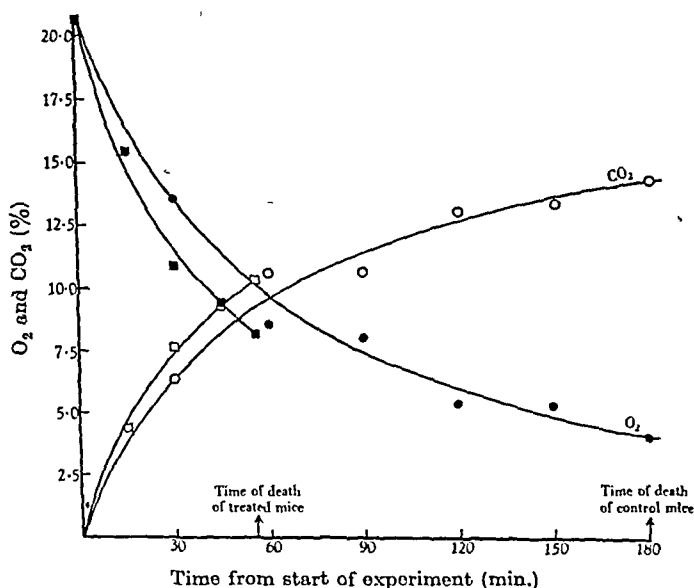


FIG. 3. Oxygen and carbon dioxide content of enclosed air at successive stages of experiment. Each point shows the average for five separate jars. Normal mice: ● O<sub>2</sub>, ○ CO<sub>2</sub>. Mice injected for one week with dried thyroid: ■ O<sub>2</sub>, □ CO<sub>2</sub>.

*Progressive changes in composition of air during experiment.* Thirty normal male mice were used. Samples of air (50 ml.) were taken from each of five jars after 30, 60, 90, 120 and 150 min., and at the time of death. Only one sample was taken from each jar. The mean oxygen percentage and carbon dioxide percentage at each observation is shown graphically in Fig. 3, with curves fitted by eye. The oxygen

content fell rapidly in the early stages of the experiment, and then more slowly; reciprocally, the carbon dioxide content rose rapidly at first and then more slowly.

*Effect of temperature on composition.* The effect of temperature on the composition of the enclosed air was investigated to determine whether the prolongation of life at lower temperatures was due to decreased oxygen consumption, or to increased resistance to oxygen lack.

Ten normal female mice were enclosed at a room temperature of 19° C. Air samples were taken after 120 min. from five jars, and at the time of death from the other five jars. The procedure was repeated with another ten normal female mice at 23° C.

The results given in Table 3 show that after 2 hr. there was little difference in the composition of the air at the two temperatures, but that slightly, although not statistically significantly lower oxygen and higher carbon dioxide percentages were reached before death occurred at 19° C. than at 23° C. The M.S.T. at 19° C. was almost double that at 23° C., and highly significantly different from it ( $t = 5.35$ ,  $P < 0.01$ ).

Table 3. *Effect of room temperature on survival and composition of the enclosed air*

Room temp. ° C.	Time	Mean CO <sub>2</sub> percentage ± S.E. of mean	Mean O <sub>2</sub> percentage ± S.E. of mean
19	After 2 hr.	13.2 ± 0.57	5.2 ± 0.62
	At death (299 ± 23 min.)	14.6 ± 0.26	3.5 ± 0.43
23	After 2 hr.	13.0 ± 0.23	5.5 ± 0.34
	At death (156 ± 13 min.)	13.75 ± 0.52	4.5 ± 0.29

It may be concluded that lowering the environmental temperature does not lower the initial rate of oxygen consumption but may slightly increase resistance to anoxia. Perhaps because of the progressive decrease in consumption (implied by extrapolating the curves in Fig. 3), a much longer S.T. is seen in mice kept at 19° C.

*The effect of absorbing carbon dioxide and moisture.* Bert concluded from his experiments that at pressures between 1 and 2 atm. death in closed vessels was due largely to lowered oxygen tension, but that the rise in carbon dioxide tension was a contributory factor.

We therefore investigated the effect of absorbing carbon dioxide. In our first experiment three groups of normal female mice were used. In group I each of the ten jars contained a tube of soda lime to absorb carbon dioxide and moisture. In group II each jar contained a tube of calcium chloride to absorb moisture alone. In the controls, group III, each jar contained a tube of crystalline sodium sulphate. The results (Table 4) show that, whereas absorbing the moisture alone had little effect, removal of the carbon dioxide and moisture greatly prolonged the M.S.T. of the mice.

Table 4. *Effect of absorbing CO<sub>2</sub> and moisture*

		Survival time of ten normal female mice (min.)	
		Range	Mean ± S.E. of mean
Group I	Soda lime	212-329	255 ± 12
Group II	Calcium chloride	145-190	159 ± 4
Group III	Sodium sulphate	147-260	189 ± 11

A similar experiment, but omitting the calcium chloride group, was carried out on forty female mice using jars of two different sizes (1 and 2 lb.). The results, summarized in Table 5, show that the effect of absorbing the carbon dioxide was to prolong the M.S.T. in both sizes of jar, the increase being relatively much greater at the 1 lb. size than at the larger one.

Table 5. *Effect of size of jar and absorption of CO<sub>2</sub>*

Size of jar	Mean survival time $\pm$ S.E. of mean (min.)	
	With soda lime	With sodium sulphate
1 lb.	178 $\pm$ 7	88 $\pm$ 4
2 lb.	260 $\pm$ 16	165 $\pm$ 7

No. of mice = 10 per group.

The effect of absorbing the carbon dioxide and moisture on the rate of utilization of oxygen and on the terminal oxygen percentage are further discussed on pp. 199, 203.

#### SURVIVAL TIME OF MICE TREATED WITH DRIED THYROID OR IODINATED PROTEIN

##### *Preliminary experiments*

*Dried thyroid powder.* Preliminary investigations to determine whether treatment with thyroid preparations would influence the s.t. of mice in closed vessels were carried out with a crude dried thyroid powder (Batch 628,423). Ten male mice received, on four successive days by injection, 1 ml. of saline suspension containing approximately 5 mg. of the powder. Two days after the last injection the mice were tested in comparison with ten normal uninjected male mice. The M.S.T. of the group of ten treated mice was 71 min., the individual values ranging from 50 to 105 min.; the M.S.T. of the control group was 124 min., the range being from 105 to 150 min. In another experiment, ten male mice received seven daily injections of 5 mg. of the dried thyroid powder in 1 ml. of saline, and ten control mice received seven daily injections of 2 mg. of casein in 1 ml. of saline. Four days after the last injection one thyroid-injected mouse and one casein-injected mouse were placed together in each of ten 3 lb. jars, at a room temperature of 24° C. In each jar the thyroid-treated mouse survived for a much shorter time than the casein-injected control, and the M.S.T. of the ten thyroid-injected mice (48 min.) was less than half that of the casein-injected controls (112 min.). In considering these results it must be remembered that the change in the composition of the enclosed air must have been slower after the death of the first mouse.

*Crude ox thyroglobulin.* Forty normal female mice were used, divided into four groups of ten. Each mouse was given six daily injections of 1 ml. of a solution of Ox Thyroid II (see p. 188), the mice in group I receiving 2 mg. daily, those in group II 1 mg. daily, and those in group III 0.5 mg. daily. Those in group IV received 2 mg. of casein daily as controls.

Two days after the last injection the mice were enclosed at a room temperature of 23.5–24.5° C. and the M.S.T. for each group was calculated. The results are shown in

Fig. 4 (circles), with the dosage plotted horizontally on a logarithmic scale and the M.S.T. in minutes plotted vertically. There is an obvious linear relation between the M.S.T. and the log dose.

*Iodinated casein.* The influence of treatment with iodinated casein on the S.T. of female mice was investigated using fifty mice, divided into five groups of ten.

Solutions were prepared containing 4, 2, 1 and 0.5 mg./ml. of iodinated casein, and also a solution containing 2 mg./ml. of casein. The mice received three injections of one of the solutions on alternate days. Three days after the last injection the S.T. were determined. The M.S.T. are shown in Fig. 4 (squares), plotted similarly to those for the mice receiving Ox Thyroid II. A close linear relationship is again seen between the log dose and the M.S.T.

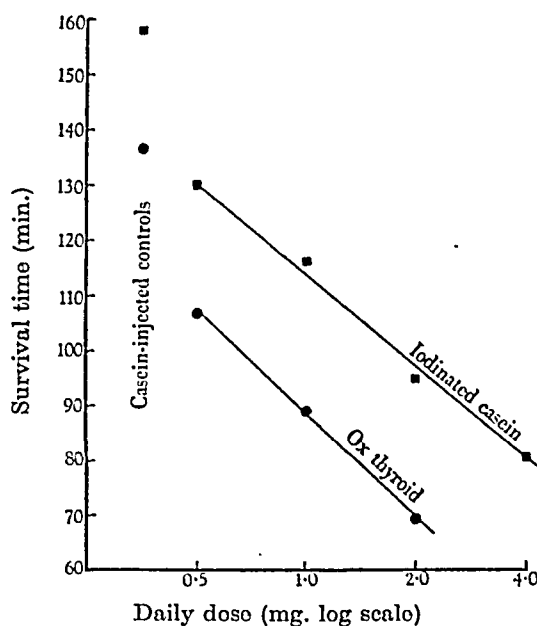


FIG. 4. Relation between survival time and dose of ox thyroid or iodinated casein. Each point shows the average for ten mice.

#### *Duration of treatment*

Both Gaddum & Hetherington [1931] and Mørch [1929] found that the increase of carbon dioxide production caused by daily oral treatment with thyroid gland preparations reached a maximum at the earliest in 10 days, and at the latest in 3 weeks. It seemed possible, therefore, that a similar duration of treatment would be necessary to obtain a maximal effect on the survival of mice in closed vessels. An experiment was designed to compare the effect of 1, 2 and 3 weeks' treatment with iodinated casein on the S.T. of mice in 2 lb. jars. 180 young female mice of approximately the same age, and weighing between 16 and 18 g., were divided at random into three groups of sixty. In the first week of the experiment only mice in group A were treated; in the second week those in groups A and B; in the third week those in groups A, B and C were treated. The weekly treatment consisted of three injections of iodinated casein on alternate days. Each group of sixty mice was divided at random into five sets of twelve mice. The mice in one set from each of the three groups were injected with 2 mg. of casein. The remaining four sets in each group were given graded doses of iodinated casein, 4.0, 2.0, 1.0 or 0.5 mg. per injection.

Treatment of all the mice finished on the same day, and the survival test was carried out on the third day after the last injection. Ten mice were chosen at random from each set of twelve, the spare animals being discarded. The M.S.T. was determined for each of the fifteen sets. The results are presented graphically in Fig. 5, which shows the relation between dose and response for each duration of treatment. The curves obtained with mice which had received treatment for 2 weeks and 1 week respectively are closely similar. With mice treated for 3 weeks the response to the

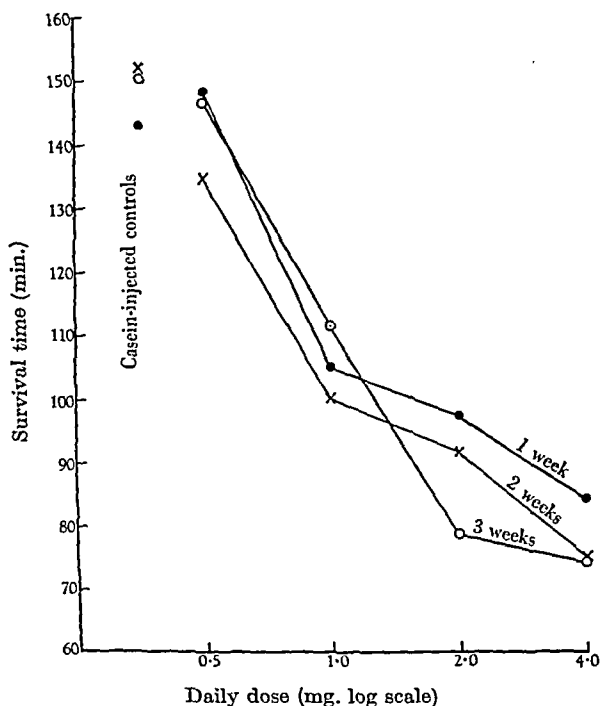


FIG. 5. Effect on survival time of duration of treatment with iodinated casein. Each point shows the average of ten observations.

4 mg. dose was the same as for group B, but an inexplicable difference in the shape of the curve was obtained. The joint departure of these curves from linearity is statistically significant ( $P < 0.05 > 0.01$ ). This is not the only instance of such departure encountered in these tests (see p. 204). It will be seen that the three curves are substantially similar in slope and position, and it may be concluded that a satisfactory and easily measurable response could be obtained with a course of treatment lasting for 1 week only, and that no marked intensification of the effect was obtained by prolonging treatment.

A further experiment was therefore carried out to determine whether effective treatment could be obtained by a single injection. Eight groups of ten mice were used. On 4 successive days, two groups of ten mice each were injected subcutaneously, one group with 4 mg. of iodinated casein, and one group (controls) with 4 mg. of casein. On the fifth day of the experiment the S.T. of the eight groups of mice were determined. The results showed that a single injection of iodinated casein reduced the M.S.T. of



mice tested up to 4 days afterwards, as compared with that of casein-treated control animals.

Confirmation of this result was obtained in another experiment in which male mice were tested 2 or 3 days after a single injection of 1 or 2 mg. of Ox Thyroid II. Table 6 gives the results of this experiment.

Table 6. *Effect in ten male mice of a single injection of ox thyroid*

Treatment	Mean survival time $\pm$ s.e. of mean (min.)	
	2 days after injection	3 days after injection
1 ml. saline	139 $\pm$ 21	115 $\pm$ 12
1 mg. Ox Thyroid II	86 $\pm$ 6	97 $\pm$ 7
2 mg. Ox Thyroid II	71 $\pm$ 3	83 $\pm$ 4

*Interval between the last injection and the test*

It was important to ascertain the optimal interval between the last injection with iodinated casein and the test of s.t. For this purpose four groups each of sixty mice were used. Group I were controls, and received three subcutaneous injections, on alternate days, of 2 mg. of casein. Groups II, III and IV received similar treatment with iodinated casein in doses of 1, 2 and 4 mg. respectively. All the mice were injected synchronously. On the first, second, fourth, eighth, and sixteenth days after the last injection the M.S.T. was determined for each group.

The results are shown in Fig. 6. Good dose-response lines were obtained in the mice tested 1, 2, 4 and 8 days after the last injection. The lines were parallel and showed clearly that the maximum effect of a week's treatment occurred on the second day after the last injection. At 1 day after the effect had not developed fully; at 4 and 8 days after it was passing off. At 16 days after the end of treatment no trace of the effect remained at any one of the dose levels.

The experiments described in the previous section had shown that there was little difference between results obtained on the second and third days after the last injection, the effect usually being slightly greater on the second day.

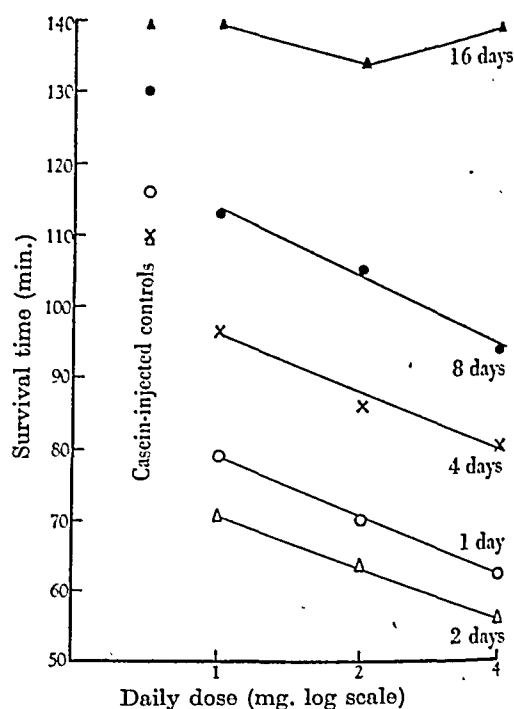


FIG. 6. Effect on survival time of interval between test and end of treatment with iodinated casein.

*Changes in the enclosed air*

*Composition of air at death of thyroid-treated animals.* The composition of the enclosed air at the time of death of thyroid-treated mice and of casein-treated control mice was determined to provide information as to the immediate cause of the earlier death of the hyperthyroidic animals. Five male mice were injected subcutaneously

with 5 mg. of dried thyroid daily for 6 consecutive days. On the same days, five control mice within the same weight range were similarly given 5 mg. of casein. On the day after the last injection the mice were tested at 22° C., and air samples taken for analysis from each jar when its occupant died. The results, summarized in Table 7, show that the thyroid-treated mice died with the oxygen percentage about 4% higher, and the carbon dioxide percentage about 4% lower than was found at the time of death of the control mice. It seemed, therefore, that decreased resistance to anoxia or to carbon dioxide poisoning must be at least a factor in the earlier death of the thyroid-treated animals. The possibility that an increased metabolic rate was also concerned was then investigated.

Table 7. *Composition of enclosed air at death of thyroid-treated mice and of casein-treated controls*

	Survival time (min.)		CO <sub>2</sub> percentage		O <sub>2</sub> percentage	
	Range	Mean $\pm$ s.e.	Range	Mean $\pm$ s.e.	Range	Mean $\pm$ s.e.
Thyroid-treated mice	50-60	54 $\pm$ 2	8.9-11.8	10.11 $\pm$ 0.47	7.23-9.76	8.56 $\pm$ 0.40
Control mice	145-255	175 $\pm$ 21	13.8-14.5	14.27 $\pm$ 0.86	3.9-5.6	4.56 $\pm$ 0.30

Values are the averages for groups of five male mice  $\pm$  s.e. of mean.

*Progressive changes in composition.* The rate of metabolism following thyroid treatment was first studied in twenty male mice which had received six daily injections of 5 mg. of the dried thyroid preparation, and were tested on the third day after the last injection. At 15, 30 and 45 min. after closing the jars air samples were taken for analysis, five jars being sampled at each stage. The remaining five jars were sampled at the time of death of the mice. The results are shown in Fig. 3, in comparison with those for the untreated mice referred to on p. 191. The two curves showing the decrease in oxygen content of the enclosed air for treated and control mice are almost identical in slope, as are the two showing the increase in carbon dioxide content. The slightly steeper slope of the curves for the treated mice is of doubtful significance, especially as the two groups of mice were tested on different days. The striking feature of the results is the curtailment of the curves for the thyroid-treated mice due to early death at an oxygen percentage 4% higher and a carbon dioxide percentage 3% lower than was reached in the case of the controls. It may be concluded that, under the conditions of this experiment, the reduced survival time of the thyroid-treated mice was due mainly to their increased sensitivity to anoxia or to carbon dioxide excess, rather than to an increased metabolic rate.

The experiment was repeated on a group of fifteen mice which had been injected with 4 mg. of iodinated casein on alternate days for 3 weeks, and a control group similarly treated with casein. The test was carried out on the third day after the last injection. Air samples were taken for analysis after 20 min., 40 min. and at death, from the jars containing mice treated with iodinated casein, and after 20, 40, 60 and 80 min., and at death, from the jars containing control mice. Five jars from each group were sampled at each of these times. The mean values for the oxygen percentage and the carbon dioxide percentage are shown in Fig. 7. The points for the oxygen content for the two groups of animals are so close that only one curve can be drawn.

The same applies to the points for carbon dioxide content. The only difference, as in the previous experiment, is the sudden termination of the lines for the treated mice owing to their early death. It would appear, therefore, that even prolonged treatment with iodinated casein fails to give a rise in metabolic rate detectable by the present technique, and that the early death of treated mice is due solely to their increased sensitivity to anoxia or carbon dioxide poisoning. This increased sensitivity appears rapidly and is not accentuated by prolonging treatment for 3 weeks.

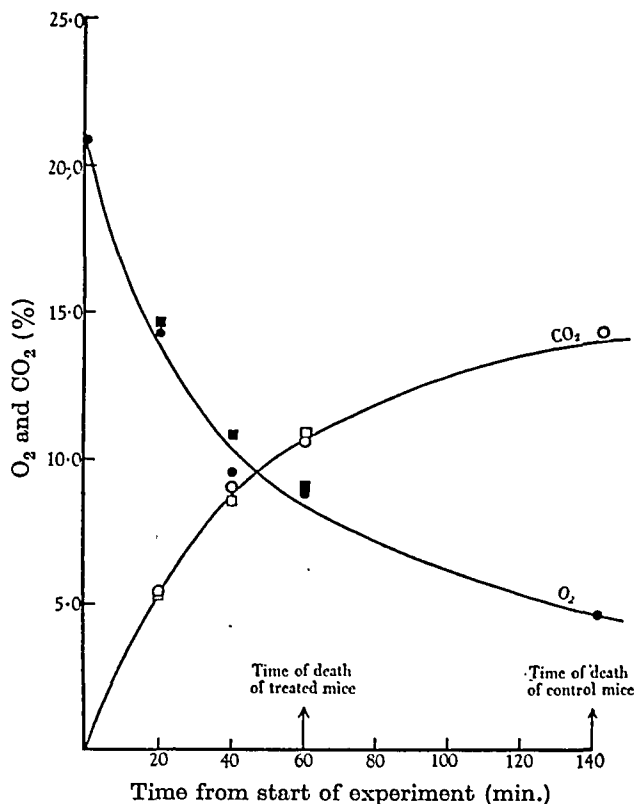


FIG. 7.

FIG. 7. Oxygen and carbon dioxide content of enclosed air at successive stages of experiment. All mice were injected for 3 weeks with iodinated casein or casein. Each point shows the average of five observations. The curves are fitted by eye to points for control mice. Casein (control): ●  $O_2$ , ○  $CO_2$ . Iodinated casein: ■  $O_2$ , □  $CO_2$ .

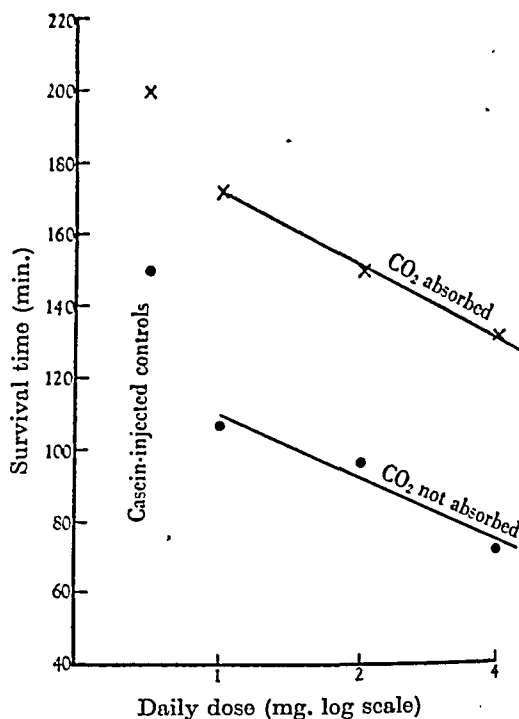


FIG. 8.

FIG. 8. Effect of absorbing carbon dioxide on response to iodinated casein. Each point shows the average of ten observations.

### *Effects of absorbing carbon dioxide and moisture*

*Survival time of mice treated with iodinated casein.* As with untreated animals (see p. 192), mice injected with iodinated casein survive much longer in closed vessels if the carbon dioxide and moisture are absorbed. In Fig. 8, the upper line shows the M.S.T. of groups of ten female mice treated with different doses of iodinated casein and tested in the presence of soda lime. The lower line shows the M.S.T. of groups of ten similar female mice tested simultaneously without soda lime. At each dosage level, as with the casein controls, removal of carbon dioxide prolonged the S.T. by about 60 min., so that the two lines are parallel. (Other similar results, together with a detailed statistical analysis, are given below on p. 203.)

*Rate of change of oxygen content of enclosed air.* The fact that s.t. for both normal and treated mice was much greater in the absence of carbon dioxide was investigated by determining the rate of change in the oxygen content of the enclosed air in the absence of carbon dioxide. Three subcutaneous injections of 4 mg. of iodinated casein were given to male mice and their survival time, in comparison with that of mice similarly injected with casein, was determined 3 days after the last injection. The m.s.t. was 162 min. for the mice treated with iodinated casein, and 252 min. for the casein-injected controls; the mean oxygen percentages at death were 6.7 and 4.0 respectively. The decrease in the oxygen content of the air was slightly greater for the mice treated with iodinated casein than for the controls (Fig. 9), but not sufficiently so to indicate any important difference in metabolic rate.

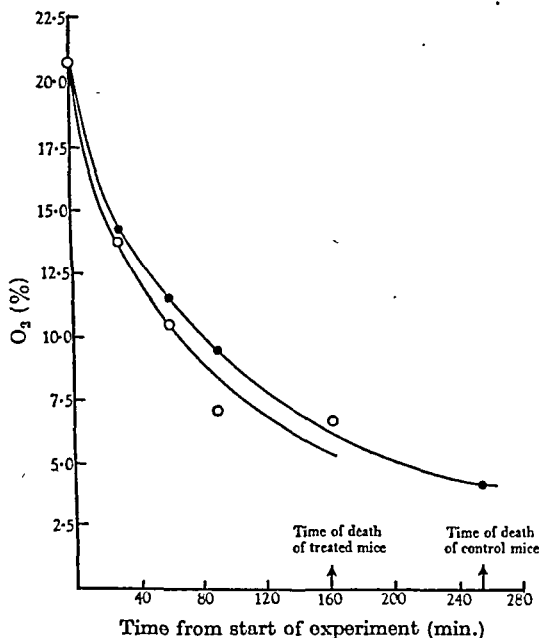


FIG. 9.

FIG. 9. Oxygen content of jars at successive stages of experiment on treated and control mice with carbon dioxide absorbed. Each point shows the average of five observations. Casein (control), ●; iodinated casein, ○.

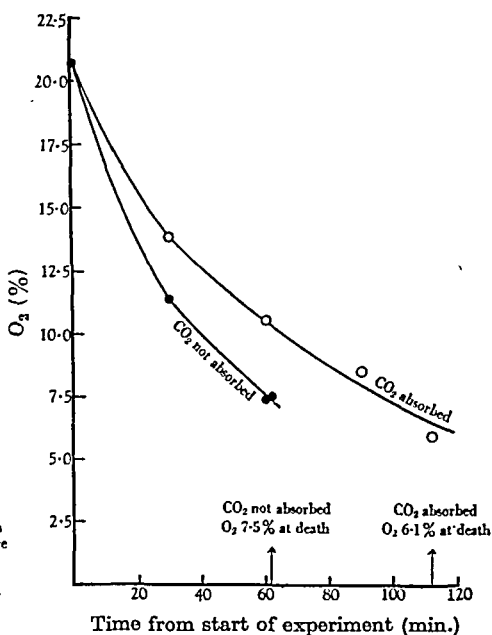


FIG. 10.

FIG. 10. Effect of absorbing carbon dioxide on oxygen consumption and survival time in mice treated with iodinated casein. Each point shows the average of five observations.

In another experiment the rate of oxygen decrease in the enclosed air, and the oxygen percentage at the death of male mice treated with iodinated casein tested with and without soda lime were compared. All the mice were given three subcutaneous injections of 4 mg. of iodinated casein on alternate days, and the experiment performed 3 days after the last injection. The results are shown in Fig. 10. Two conclusions may be drawn: (1) the rate of oxygen consumption was considerably more rapid in the presence than in the absence of carbon dioxide; (2) the level at which the oxygen content would not support life was higher when carbon dioxide was allowed to

accumulate than when it was absorbed. These conclusions are in keeping with the fact that excess carbon dioxide acts as a respiratory stimulant and that carbon dioxide poisoning *per se* is probably a factor in the death of incarcerated animals.

#### OTHER VARIABLES ASSOCIATED WITH THE TEST

##### *Size of mouse*

The influence of body weight on the s.t. of normal mice in closed vessels was investigated on normal female mice ranging from 14 to 29 g. A definite relationship between body weight and s.t. was found, the small mice tending to live longer than the heavier ones (Fig. 11). There was a considerable individual variation in s.t. at any given weight, and within the group of mice of 20–24 g. there was no obvious correlation between body weight and s.t. The overall result is not surprising; although small animals have a higher B.M.R. than larger animals of the same species, a larger animal uses a greater total amount of oxygen in a given time. Furthermore, the conditions of this test were not basal.

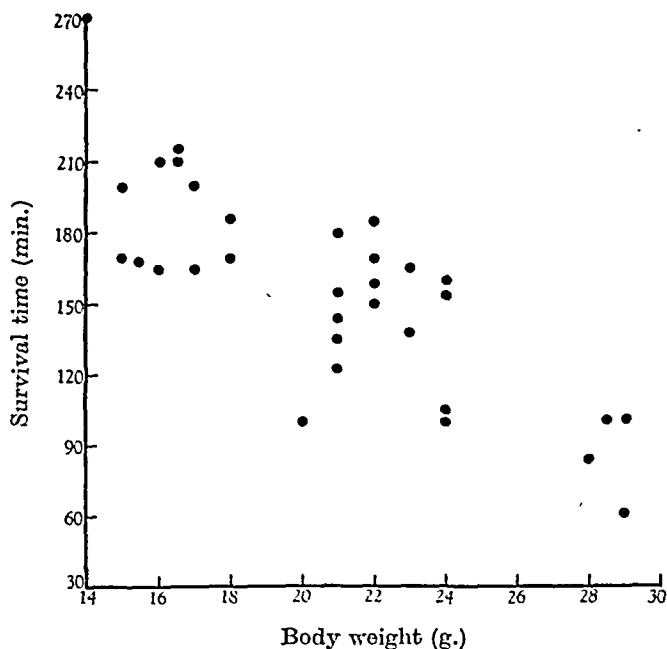


FIG. 11. Effect of body weight on survival time of normal mice.  
Each point is for a single mouse.

As a practical matter, selection of treated mice on a weight basis presented some difficulty. If animals were selected to be of the same weight at the beginning of treatment, they would differ at the end, because those most sensitive and those most heavily dosed would tend to lose weight, and vice versa. Elimination of those below and above certain weights might remove the best and the worst reactors. In practice we found that, during treatment for 1 week with iodinated casein or control material, mice tended not to gain or lose more than 2 g. Our routine method has therefore been to select an appropriate total number of mice 18–20 g. in weight and distribute them at random to receive the control substance and the dosage levels of the preparation under investigation. No further selection by body weight was made,

and if there were any extra mice at the time of test those to be discarded were picked out by drawing lots. Each mouse was weighed immediately before being bottled. The influence of body weight on s.t. was found to be unimportant in the range of weights so obtained. The results are discussed more fully below (p. 203).

### *Sex of mouse*

The possibility of a sex difference in the s.t. of mice in closed vessels was studied in five groups of mice each comprising ten males and ten females. The doses used were 2 mg. of casein, and 0.5, 1.0, 2.0 and 4.0 mg. of iodinated casein for each of three subcutaneous injections on alternate days. The s.t. was determined on the third day after the last injection. The results are summarized in Fig. 12, which shows separate

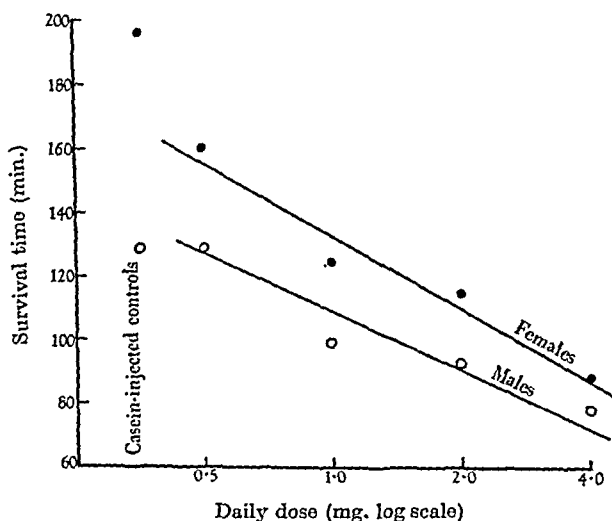


FIG. 12. Effect of sex on survival time of control and treated mice. Each point shows the average for ten mice.

dose-response curves for males and females. There is no difference in slope of the dose-response curves with the two sexes, but the males are considerably more sensitive. The females are more variable (see also p. 204), probably because of their being in various stages of the oestrous cycle, which is known to influence metabolic rate [Brobeck, Wheatland & Strominger, 1947].

### *Temperature during treatment*

There is evidence that the activity of the thyroid gland of mice varies according to the environmental temperature at which they live, and it seemed therefore that environmental temperature in the days preceding test (as well as during test) might be an important factor in s.t. Mice were injected on three alternate days with casein or iodinated casein, half being kept during the week of treatment in a heated room at 20–22° C. and half in an unheated room at 9–12° C. The results (Table 8) do not suggest that environmental temperature during treatment is of any importance.

Table 8. *Effect of environmental temperature during treatment*

Environmental temperature during week of treatment	Mean survival time (min. $\pm$ s.e.) after injection of		
	2 mg. casein	2 mg. iodinated casein CB 3/62	4 mg. iodinated casein CB 3/62
20-22° C.	166 $\pm$ 17	94 $\pm$ 6	83 $\pm$ 5
9-12° C.	124 $\pm$ 14	87 $\pm$ 4	78.5 $\pm$ 6

No. of mice = 5 per group.

#### USE OF THE CLOSED VESSEL TECHNIQUE FOR THE ASSAY OF THYROIDAL ACTIVITY

##### *Relative importance of the chief variables*

In order to decide on the best conditions for the routine use of the closed vessel technique in assaying preparations of unknown activity, factorially designed tests were made. These tests included some of the factors already discussed above. The application of this type of factorial test preferably follows preliminary work, as the test may fail because of insufficient knowledge of the range of effective doses and conditions. Factorial tests involve the simultaneous investigation of several variables; every possible combination of all variables at all levels is included in the same test. This does not, however, mean that vast numbers of animals must be employed, as the structure of the test is such that the effect of each variable is as fully evaluated as if the entire test were devoted to the study of it alone [Fisher, 1947]. In addition, the interactions of the various treatments included in the test are available for study, which would not be the case without factorial design.

A test designed to explore the effect of using 1 and 2 lb. jars with or without absorbents on the dose-response line of female mice was set up in three replications, each occupying one-half of a day, the whole test being completed in 2 days (exclusive of injections). In this test, three injections were given, one on each of three alternate days, as described above (p. 194). The treatments were the following.

(i) Injection with 0.5, 1.0, 2.0 or 4.0 mg. of iodinated casein or 1.0 mg. of casein alone.

(ii) The use of 1 or 2 lb. jars.

(iii) The use of no absorbent or of soda lime or calcium chloride.

Each replication employed one mouse on each of the 30 possible combinations of treatments. The results are shown in Table 9, and the corresponding analysis of variance in Table 10. Owing to the loss of one result, the degrees of freedom available for the estimate of error are reduced to 51 instead of 52; our estimate of the missing result was obtained by the method of Snedecor [1946]. (The main effects and first order interactions are isolated; all other interactions together form the error term.)

Highly significant effects are seen from variations in dosage, jars and absorbents, but the absence of significant replication/absorbent, dose/absorbent, and jar/absorbent interactions shows that the effect of absorbents was the same in all circumstances. The absence of significant variation attributable to replication, or to replication/dose interaction shows stability of the responses. On the other hand, a significant, although not highly significant, replication/jar interaction indicates that the differential effect of jar size was not completely stable from test to test.

Table 9. *Individual survival time in minutes in the three replications of a factorial test of the influence of jar size and absorbents in the response of mice to iodinated casein*

Jar size	Replication ... Absorbent	Dose of iodinated casein (mg.)															Totals
		0.0			0.5			1.0			2.0			4.0			
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
1 lb.	None	61	64	78	59	67	65	55	45	54	58	52	39	56	34	53	840
	CaCl <sub>2</sub>	56	50	48	55	61	56	58	50	72	63	56	62	55	39	52	833
	Soda lime	148	153	110	110	118	90	108	86	90	68	84	70	60	74	86	1455
	Totals	265	267	236	224	246	211	221	181	216	189	192	171	171	147	191	3128
2 lb.	None	136	183	271	105	177	143	147	110	108	93	96	81	80	64	78	1872
	CaCl <sub>2</sub>	135	177	179	94	139	(150)*	79	180	115	97	172	101	115	80	94	1907
	Soda lime	155	207	337	278	292	185	143	208	166	137	215	142	87	163	177	2892
	Totals	426	567	787	477	608	478	369	498	389	327	483	324	282	307	349	6671
	Totals		2548			2244			1874			1686			1447		9799

\* Estimated replacement for lost datum.

Table 10. *Analysis of variance for the data of Table 9*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P*
Main effects:					
Between replications	2	5,312	2,658	3.1	> 0.05
Between doses	4	42,891	10,723	12.4	< 0.001
Between jars	1	139,475	139,475	161.2	< 0.001
Between absorbents	2	58,404	29,202	33.7	< 0.001
Interactions:					
Replication/dose	8	11,565	1,446	1.7	> 0.05
Replication/jars	2	7,119	3,560	4.1	< 0.05
Replication/absorbents	4	2,087	521	0.7	> 0.05
Dose/jars	4	12,295	3,074	3.6	< 0.05
Dose/absorbents	8	9,567	1,196	1.4	> 0.05
Jar/absorbents	2	3,307	1,654	1.9	> 0.05
Error	51	44,139	865		

\* The probability that the observed effects are due to chance.

The remaining significant interaction—dose/jars—shows that the relation between responses to the different doses was not the same with different jar sizes. In fact, the slope is steeper with the 2 lb. jars. No significant improvement was seen on adjusting responses for body weight by covariance analysis.

This test therefore indicated that it is no advantage to use absorbents, but that 2 lb. jars are preferable to 1 lb. jars. (The error of a single determination decreased with a decreasing M.S.R., but this was not sufficient to counteract the gentler slope with 1 lb. jars.) It also showed that undesirable interactions are negligible if one type of jar is used.

A further test was therefore set up to confirm these findings, if true, and to explore more accurately the suitability of the dose/response line for assay work. Since the only effective absorbent was soda lime, this was tested against no absorbent, in 2 lb. jars, using ten groups, each of five female mice, and four dose levels with controls. The results are given in Table 11 and the analysis of variance in Table 12.

The results entirely confirm those of the first factorial test and show that the dose/response relationship in this test is entirely satisfactory as regards slope and linearity. We are now virtually certain that there is no point in using absorbents. Once more, no significant dependence of response on body weight was seen in this test.



Table 11. *Mean survival time in minutes in groups of five female mice with and without the absorption of carbon dioxide in 2 lb. jars*

Absorbent	Dose of iodinated casein (mg.)				
	0.0	0.5	1.0	2.0	4.0
Nil	155.6	166.8	154.8	95.2	82.2
Soda lime	293.4	237.2	201.4	172.6	154.8

Table 12. *Analysis of variance for the data of Table 11*

Source of variation	Degrees of freedom	Mean square	F	P
Differences between control groups	1	47,472	30.5	< 0.001
Differences between dosage groups	1	41,281	26.5	< 0.001
Differences between controls and dosage groups	1	36,585	23.5	< 0.001
Slope of combined dose/response line	1	47,957	30.8	< 0.001
Departure from parallelism of the two lines	1	7	All non-significant	
Various types of departure from linearity	1	31		
	1	51		
	1	972		
	1	1,255		
Error	40	1,556		

The difference between the responses and variability of males and females reported on p. 201 was the subject of the next factorial test. The results have already been presented; the analysis of variance is given in Table 13. The important features of this test are the highly significant sex differences in sensitivity and variance, and the absence of a significant difference in slope. Clearly, it is preferable to use males for assaying preparations. However, the departure of the dose/response lines from linearity (of the same type in both sexes) is disturbing. It does not usually occur, but in the two instances seen (the other is referred to on p. 195) this departure consists in a shorter survival time of mice injected with one of the central doses than would be expected from the other responses. No explanation for this occasional phenomenon is apparent.

Table 13. *Analysis of variance for the test illustrated in Fig. 12*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
<b>Females:</b>					
Between replications	1	208	208	0.3	> 0.05
Between doses:					
Linear regression	1	17,766	17,766	23.1	< 0.001
Deviations from regression	3	48,346	16,115	21.0	< 0.001
Replication/dose interaction	4	6,286	1,572	2.1	> 0.05
Error	40	30,734	768	—	—
<b>Males:</b>					
Between replications	1	599	599	2.5	> 0.05
Between doses:					
Linear regression	1	13,009	13,009	55.2	< 0.001
Deviations from regression	3	7,681	2,560	10.9	< 0.001
Replication/dose interaction	4	1,550	389	1.7	> 0.05
Error	40	9,428	238	—	—
Between sexes	1	20,478	20,478	40.1	< 0.001

*Method of test adopted*

The technique proposed as suitable for assays is as follows.

Four groups are used, each of twenty male mice weighing between 18 and 20 g. Two of the groups receive two different doses of the substance to be used as a standard preparation; the other two groups receive two different doses of the unknown in the same ratio. The substances are given by subcutaneous injection on alternate days, and the test is performed at 23° C. on the second or third day after the last injection. Each mouse is placed in a separate jar of the 2 lb. size. The washers are fitted round the necks of the jars, the glass lids placed in position, and the metal bands screwed down. This final action is the start of the experiment for the purpose of recording survival times. The jars are placed on their sides in rows and the survival time of each mouse recorded in minutes. The relative potency of the two substances is calculated by the usual statistical methods.

With the slope and variation in response encountered in this laboratory, such a test should give determinations of relative potency with fiducial limits of error ( $P=0.05$ ) not far exceeding about 70–140%, as long as the criteria for parallel rectilinearity of the two dose/response lines are satisfied.

## SUMMARY

1. Normal mice, confined in closed vessels of 870 ml. capacity, at 23° C., at normal pressure, survive about 2 hr. The survival time is influenced by the size and sex of the mice and by environmental temperature, and is subject to considerable day-to-day variation. It is not directly proportional to the size of the vessel.

2. Death of normal mice occurs when the concentration of oxygen in the enclosed air is down to about 5% and that of carbon dioxide up to about 13%. Absorption of carbon dioxide greatly prolongs survival, mainly by decreasing the rate of oxygen consumption, but partly by increasing resistance to anoxia.

3. Mice treated with large doses of dried thyroid preparations or of iodinated casein survive in closed vessels only about half as long as normal mice. There is a clear-cut relation between dose and survival time. The effect is not increased by prolonging treatment. It disappears in about 2 weeks. It is much influenced by the time between the end of treatment and the time of test, but is little influenced by the duration of treatment with a constant daily dose.

4. Death of treated mice occurs when the concentration of oxygen in the enclosed air is down to about 8% and that of carbon dioxide up to about 10%. Death is thus expedited by decreased resistance to anoxia rather than by increased consumption of oxygen.

5. A new method of assay of thyroidal activity has been elaborated.

We are greatly indebted to our colleague Dr F. C. MacIntosh for information and assistance.

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# A COMPARISON OF THE GROWTH OF THE OVUM AND FOLLICLE IN NORMAL RHESUS MONKEYS, AND IN MONKEYS TREATED WITH OESTROGENS AND ANDROGENS\*

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The ovum of placental mammals completes its growth long before the follicle, in which an antrum develops only after the ovum has reached full size. In the first quantitative demonstration of this fact, Brambell [1928] showed that the development of the follicle relative to that of the oocyte may be divided into two phases. In the first the regression line relating ovular to follicular size is very steep, and in the second it is practically horizontal. The point at which the two lines intersect gives the approximate time, in relation to follicular size, at which the ovum completes its growth. Measured in these terms, the time is much the same in different species, a fact which is related to the comparatively small differences in the size of the mature ovarian oocyte in all placental mammals. Thus, the diameter of the follicle at the end of its first phase of growth is only  $110\mu$  in the lesser shrew, an animal weighing 5 g. [Brambell, 1935], and  $300\mu$  in the pig, an animal weighing 10,000 times as much [Parkes, 1931]. On the other hand, the subsequent growth of the follicle, as well as the size of the mature follicle, at ovulation, are highly correlated with body size [Parkes, 1931]. Thus the diameter of the mature follicle in the shrew is  $320\mu$  and in the pig  $8000\mu$ .

The existence of these readily determined relationships made it of interest to discover whether their normal character is altered when the ovary involutes under the influence of either oestrogens or androgens. At the same time it seemed useful to discover how such treatment affects the proportions of follicles of different size.

## MATERIAL AND METHODS

The ovaries of thirteen rhesus monkeys (*Macaca mulatta*), two of which had been treated with oestrone and three with testosterone propionate, were studied (Table 1). The hormones were injected intramuscularly in oil solution. Oestrone was injected daily and testosterone propionate four times weekly. In each of the two animals marked with an obelisk (nos. 225 and 214), only a single ovary was available. In all other cases both ovaries were examined.

All the ovaries were fixed in alcoholic Bouin's fluid and embedded in paraffin. They were serially sectioned at  $10\mu$  and stained with haematoxylin and eosin. Differential changes in size after removal from the body were thus avoided, in so far as they can be, by identical methods of preparation.

Taking each section in turn, a search was made, under the microscope, for measurable follicles. Polyovular follicles were occasionally seen, but not counted. In the

\* A preliminary statement on the observations reported in this paper was made at the Anatomical Society in May 1943 [Zuckerman & Green, 1946].

case of the two animals that were treated with oestrogen, the number of atretic follicles was very high and that of normally developing follicles small. As many normal follicles as possible were measured.

The section giving the largest dimensions of each follicle was selected, and the follicle, oocyte and nucleus then measured in two directions, at right angles to each other, from tracings of projected images. To obviate error due to spherical aberration, each follicle was brought to the centre of the field before tracing. The measurements for one pair of ovaries (286) were made from negative photomicrographs made directly on to bromide paper.

Table 1. *Details of animals studied*

No.	Body wt. at death (g.)	Estimated age at death* (months)	Treatment	Dose	Total administered (mg.)	Duration (months)
225†	1460	< 20	Normal control	—	—	—
252	1420	< 20	" "	—	—	—
194	2000	24	" "	—	—	—
141	2300	26	" "	—	—	—
251	3080	38	" "	—	—	—
171	4000	44	" "	—	—	—
286	3180	Mature	" "	—	—	—
390	4600	Mature	" "	—	—	—
406	2760	30	Oestrone	100 $\mu$ g. daily	3.1	1
409	3000	32	Oestrone	100 $\mu$ g. daily	4.4	1.5
193	2260	28	Testosterone pro- pionate	40–100 mg. weekly	1,955	5
214†	5600	60	" "	15–25 mg. weekly	972.5	12
439	2010	28	" "	100–200 mg. weekly	12,350	15

\* Based on dentition and body weight.

† Only one ovary examined.

These measurements, in  $\mu$ , provided data about the relative growth of the ovum and follicle, and also a rough indication of the relative numbers of follicles in normal and untreated ovaries. To obtain further information about the latter question, a survey was made of the relative numbers of follicles in different stages of growth, the stages chosen being (a) primordial ova not surrounded by granulosa cells, (b) ova surrounded by one layer of cells, (c) by two layers, (d) by three layers, (e) by four layers, (f) by six layers, and (g) follicles with an antrum. To obviate selection of follicles to be enumerated, the following procedure was adopted. All follicles at different stages of growth were counted throughout different microscopic fields selected at random, care being taken to avoid overlap of the fields studied. Counting was done from projected images, and only follicles with ova showing nuclei were taken. After being counted, each follicle was crossed off the projected picture to prevent it being counted again.

A thousand ova and follicles were counted in each of seven pairs of ovaries. The precautions taken to avoid selection did not allow of as great a number in the other six, but in none were fewer than 500 counted (Tables 3, 4).

Standard methods were followed in the statistical analysis of the results. Differences have not been regarded as significant except where the likelihood that they may have been due to chance was less than 1 in 50.

## RESULTS

*Relative growth of ovum and follicle*

Regression equations in the form  $y = a + bx$  for the sizes of ovum and follicle have been calculated for each animal, the data in each case being separated into that part which relates to the initial phase of development when both are growing, and that relating to the subsequent phase when for all practical purposes the follicle alone is growing. Measurements made in the immediate region of the point of intersection of the two regression lines, as determined by inspection of preliminary plots of the data, were not included in the computations, since any decision to regard them as belonging to the one or the other phase of development could only be arbitrary.

*Normal animals*

The results obtained for the eight normal animals are shown in Table 2, and the regression lines for phase I are given in Fig. 1.

The values for the coefficients of  $a$  and  $b$  in phase I are all highly significant ( $P > 0.01$  in all cases). So, too, are the values of  $a$  in phase II. On the other hand, only two of the values of  $b$  in this phase differ significantly from zero (no. 141, where  $P$  is  $> 0.02 < 0.01$ , and no. 171, where  $P$  is  $> 0.01$ ), and in this respect the present observations confirm the conclusion, established for other species by Brambell and Parkes, that during this phase of growth the size of the ovum is practically stationary.

Examination of the results points to several other interesting conclusions. The first is that the slope of the regression line in phase I may differ significantly in different animals belonging to the same species, and that the differences show no regular trend with age. Thus a  $\chi^2$  test shows that the values of  $b$  in phase I do not represent a homogeneous series, while comparison of the  $b$  values in pairs (i.e. twenty-eight combinations), by the method outlined in Fisher [1941], shows that in nine of the comparisons the differences in the  $b$  values are significant ( $P > 0.02$ ). Similarly, statistical analysis shows that the values of  $a$  do not constitute a homogeneous series.

A further analysis was made by subdividing the eight 'normals' into one group of three in which the condition of all the visceral organs at autopsy appeared healthier than in the other group of five. Neither subgroup constituted a homogeneous series, and their means did not differ significantly. It is concluded, therefore, that the differences observed in untreated animals between the values of  $b$  for the first phase of follicular growth represent 'individual' differences in the sense in which this expression is normally understood.

The fact that the best-fit line for phase I does not pass through the origin but always cuts the  $y$ -axis at a positive value has already been commented on by Parkes [1931], who points out in explanation that during its initial growth, the ovum is the same as the total size of the follicle, since it is not surrounded by any measurable amount of epithelium. Hence the projection of the line to a zero value of  $x$  has no meaning, whereas the initial growth of the ovum, when its size is that of the whole 'follicle', could be represented by another but very short regression line which would cut the  $x$ -axis close to zero, and which would meet the regression line for phase I at an angle.

Table 2. Coefficient of straight-line regression relationships ( $y=a+bx$ ) between size of ovum and follicle in normal and experimental rhesus monkeys

No. of animal	No. of pairs of observations	Phase I		No. of pairs of observations	Phase II		Follicle diameter at end of phase I ( $\mu$ )
		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>	
		Normal control animals					
225	108	$3.72 \pm 0.83$	$0.452 \pm 0.019$	16	$96.7 \pm 2.8$	$0.0066 \pm 0.0162$	215
252	157	$6.63 \pm 0.44$	$0.480 \pm 0.014$	57	$104.9 \pm 1.1$	$0.0049 \pm 0.0057$	205
194	61	$3.45 \pm 0.82$	$0.513 \pm 0.029$	47	$94.1 \pm 0.8$	$0.0065 \pm 0.0036$	175
141	54	$12.40 \pm 0.78$	$0.410 \pm 0.024$	41	$90.5 \pm 0.6$	$0.0116 \pm 0.0043$	195
251	121	$9.18 \pm 0.53$	$0.410 \pm 0.015$	42	$83.1 \pm 1.3$	$0.0042 \pm 0.0058$	180
171	43	$1.98 \pm 0.53$	$0.574 \pm 0.031$	89	$86.1 \pm 0.6$	$0.0138 \pm 0.0040$	150
286	90	$7.99 \pm 0.72$	$0.395 \pm 0.019$	32	$88.3 \pm 0.9$	$0.0069 \pm 0.0040$	210
390	43	$8.52 \pm 1.30$	$0.507 \pm 0.048$	63	$97.5 \pm 1.0$	$-0.0023 \pm 0.0038$	170
Oestrogen-injected animals							
406	26	$-3.57 \pm 1.32$	$0.557 \pm 0.052$	6	$107.1 \pm 4.3$	$-0.0205 \pm 0.0257$	190
409	39	$7.47 \pm 1.05$	$0.452 \pm 0.037$	16	$88.5 \pm 1.3$	$0.0115 \pm 0.0115$	185
Androgen-injected animals							
193	99	$9.34 \pm 0.62$	$0.480 \pm 0.020$	8	$89.8 \pm 1.1$	$0.0157 \pm 0.0067$	175
214	145	$16.33 \pm 0.40$	$0.368 \pm 0.011$	68	$100.5 \pm 0.8$	$0.0013 \pm 0.0071$	225
439	71	$12.61 \pm 0.79$	$0.378 \pm 0.025$	10	$94.4 \pm 1.0$	$0.0176 \pm 0.0063$	240

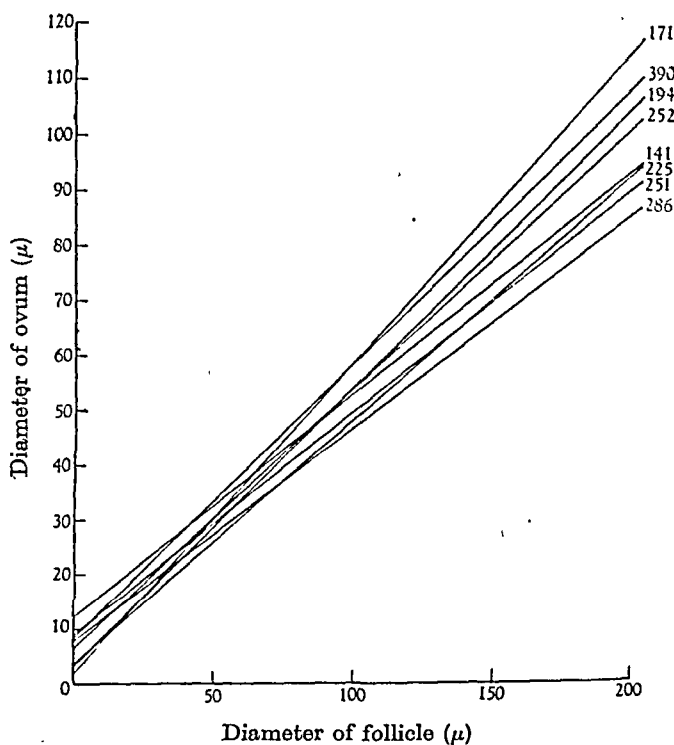


FIG. 1. Regression lines relating size of ovum and follicle in eight normal monkeys. Phase I.

Unlike phase I, the values for  $b$  in phase II represent a homogeneous series (it should be noted that in only two of the eight cases did the slope differ significantly from zero). The values of  $a$ , however, are not homogeneous.

These results clearly indicate the necessity of determining the relationship between size of ovum and follicle in several animals belonging to a species before assigning definite regression values to the species. The fact that this has not been done before prevents an accurate comparison of the present results with those obtained for other species, although it would seem that the stated values of  $b$  for phase I in the rat (0.2861) and the pig (0.1382) [Parkes, 1931], the rhinolophid bat, *R. hipposideros minutus* (0.501 and 0.596) [Matthews, 1937], and the common shrew (0.643) [Brambell, 1935], are well outside the range of variation fixed by the present results for the rhesus monkey. Values of  $b$  for the ferret and rabbit (0.5848 and 0.4730 respectively) [Parkes, 1931], for the rhinolophid bat, *R. ferrum-equinum insulanus* (0.414 and 0.501) [Matthews, 1937], and for the lesser shrew (0.49) [Brambell & Hall, 1936], appear to be within the range. So, too, does the combined value of  $b$  previously obtained for the hamadryas and chacma baboons, *Papio hamadryas* and *P. porcarius* (0.369) [Zuckerman & Parkes, 1932].

It is also difficult, for similar reasons, to make any rigid comparison between the values given for the size of the follicle at the end of phase I in different species, i.e. with the size of the follicle at which the ovum has completed its growth. The present results give a range for the untreated rhesus monkey of 150–215  $\mu$ , with a mean of 188  $\mu$ , the differences between individual animals not being related either to age or to body weight. There is a corresponding variation in the size of the fully-grown ovum (88–110  $\mu$ , mean 100  $\mu$ ).

Mean values for the coefficients  $a$  and  $b$  in the two phases of development in the rhesus monkey were computed directly from the data shown in Table 2. The separate values were not weighted in the three sets of computations where the individual values did not constitute a homogeneous series. In  $b$  of phase II they were homogeneous, and were accordingly weighted:

	Phase I	Phase II
Mean $a$	6.73 $\pm$ 1.23*	81.4 $\pm$ 5.4
Mean $b$	0.468 $\pm$ 0.022	0.0074 $\pm$ 0.0016

\* Standard error of the mean.

#### *Animals treated with oestrogen*

The data are set out in Table 2.

As in the case of the normal animals, the only values which were not significantly different from zero were those for coefficient  $b$  of phase II. The negative value for  $a$  in phase I of 406 is of interest, since it constitutes an exception to the general finding. The observation that the regression line in phase I cut the  $x$ -axis at a positive value in this animal can be related to the fact that the very small follicles in the oestrogen-treated animals were mostly pathological and undergoing atresia, and to the possibility that the primordial ova themselves were smaller than usual.

Examination showed that there was no significant difference between the two values for  $b$  of phase I. Accordingly, the original data were pooled, and the mean  $b$  value 0.504  $\pm$  0.053 obtained. This value for  $b$  is not significantly different from the  $b$  value for normal animals ( $P = 0.5 - 0.4$ ). The corresponding value for  $a$ , 1.950, also does not



differ significantly from the mean  $a$  value for phase I of the normal animals. The differences between  $a$  and  $b$  in phase II of the normal and oestrogen-treated animals were also not significant statistically.

#### *Animals treated with androgen*

The data for the three androgen-treated monkeys, also shown in Table 2, follow the usual pattern in so far as only the values for  $b$  in phase II fail to differ significantly from zero.

A  $\chi^2$  test showed that the three values for the slope of the regression line in phase I do not constitute a homogeneous series, and comparison of the  $b$  values in pairs (three combinations) shows that monkey 193 differs significantly from 214 and 439, a difference which may possibly be related to the greater duration of treatment of these two animals.

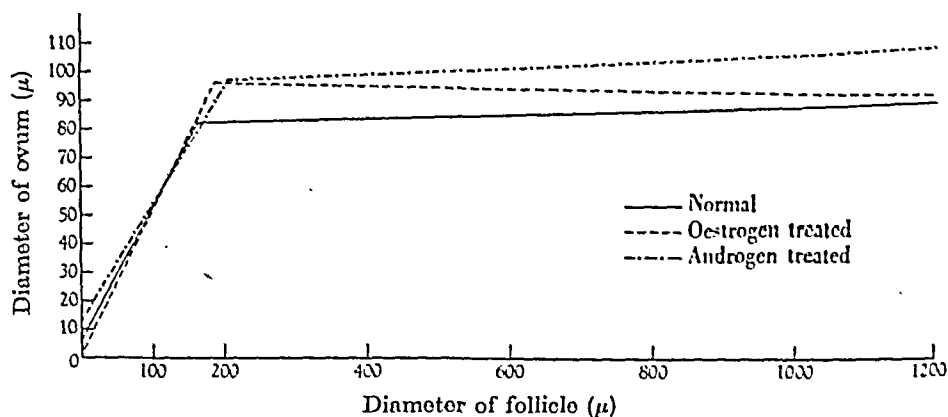


FIG. 2. Mean regression lines relating size of ovum and follicle in normal and experimental monkeys.

The mean of the three  $b$  values, not weighting the separate values, is  $0.409 \pm 0.036$ . This figure is not significantly different either from the mean value of  $b$  of phase I in the untreated monkeys, or from that of the two oestrone-treated animals.

When the comparisons are limited to the two monkeys which received the longest treatment with androgen, and which showed no statistically significant differences, similar results are obtained. Thus the weighted mean  $b$ , 0.370, for these two animals also does not differ significantly from the corresponding figures for both the untreated and the oestrone-treated monkeys.

The  $a$  values of phase I in the two animals which were under androgen stimulation for more than a year are greater than any corresponding values in the other animals studied (although statistically the difference is not significant,  $P$  being 0.5–0.2). This suggests that at the start of the follicular time scale, the primordial ova in these animals were larger than normal. The value for  $a$  (phase I) in the third androgen-treated animal is also on the high side, being exceeded by only one of the eight normal  $a$  values.

The differences between the androgen-treated and the normal and oestrogen-treated ovaries in the values of  $a$  and  $b$  of the second phase of follicular growth are not significant statistically.

The mean regression lines for both phases of ovum and follicle growth in the three groups of animals are plotted in Fig. 2.

*Conclusion*

This analysis suggests that the diminution of ovarian size in monkeys which occurs as an indirect effect of the injection of oestrogen and androgen is not associated with any marked disturbance in the character of the early growth of the ovum and follicle. The only suggestive difference found was in a greater size of the primordial ova in the androgen-treated animals at the beginning of the process of follicular development.

*Number of follicles*

While either androgenic or oestrogenic treatment reduces the size of the rhesus ovary, each has completely different effects on the structure of the organ. The ovaries of monkeys that have been treated for a prolonged period with oestrogen contain a large number of small primordial ova, a few medium-sized follicles, and an enormous number of small follicles that have degenerated, the process of destruction being most obvious in the completely 'hyalinized' ovum. Those of androgen-treated monkeys, on the other hand, may contain a far greater number of young and healthy follicles per unit of tissue than is found even in the normal ovary (Pl. 1, figs. 4-7). This immediately suggests that while they may have a similar suppressive effect on the gonadotrophic functions of the pituitary, oestrogen and androgen exert direct but opposite actions on the monkey's ovary itself. In order to pursue this point further, the procedure outlined on p. 208 for obtaining an estimate of the relative numbers of follicles in different stages of growth was followed. The results are shown in Tables 3 and 4 and Fig. 3.

Table 3. *The percentage distribution of follicles at different stages of development in normal monkeys*

Stage of follicular development	Percentage distribution in animal no.								Mean
	225	252	194	141	251	171	286	390	
0 layer(s) of granulosa cells	67.0	65.9	66.3	71.7	59.2	70.0	66.8	70.9	67.2
1       "       "	28.0	30.7	25.0	23.5	28.0	22.6	24.2	26.7	26.1
2       "       "	2.6	1.5	5.1	2.6	4.8	3.2	4.0	2.3	3.3
3       "       "	1.4	1.0	2.5	0.9	4.1	1.7	1.8	1.9	1.8
4       "       "	1.0	1.0	0.8	0.6	1.7	0.9	1.6	0.3	0.9
6       "       "	0.4	0.2	0.1	0.1	0.9	0.5	0.9	0.1	0.4
Antrum formed	0.6	0.3	0.4	0.6	1.1	0.6	0.7	0.2	0.5
No. of follicles counted	500	1000	1000	1000	1000	800	551	1014	6865

Table 4. *The percentage distribution of follicles at different stages of development in experimental monkeys*

Stage of follicular development	Oestrogen-treated			Androgen-treated			
	No. 409	No. 406	Mean	No. 214	No. 193	No. 439	Mean
0 layer(s) of granulosa cells	86.3	89.5	87.9	26.6	45.7	33.2	35.1
1       "       "	11.7	9.5	10.6	35.9	47.4	48.7	44.0
2       "       "	1.8	0.8	1.3	22.0	4.0	10.3	12.1
3       "       "	0.2	0.2	0.2	7.7	1.7	4.7	4.7
4       "       "	0	0	0	4.5	0.6	2.5	2.5
6       "       "	0	0	0	1.7	0.2	0.6	0.8
Antrum formed	0	0	0	1.5	0.3	0	0.6
No. of follicles counted	1000	1000		594	645	885	

Although the trend of observations between individual animals in the three groups appears consistent, they do not represent homogeneous series (except for the two oestrone-treated animals), when analysed by  $\chi^2$  tests. Comparison of the mean proportions for the first two stages of growth in the three groups shows, however, that the differences are highly significant statistically.

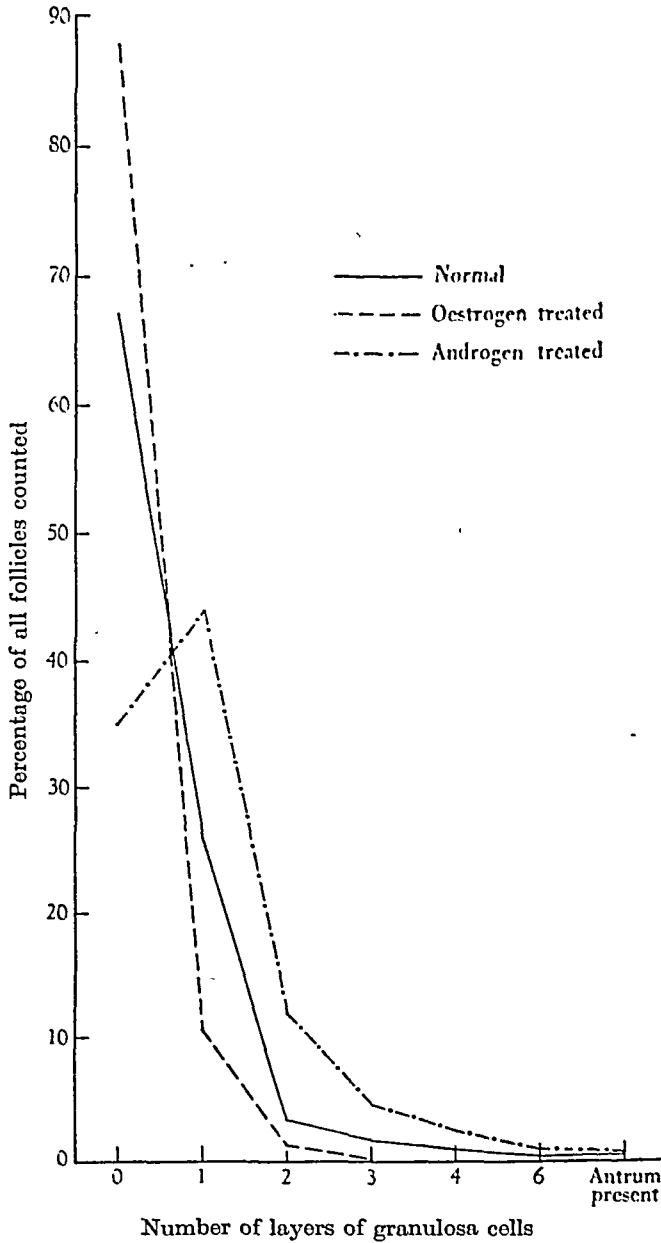


FIG. 3. The percentage distribution of follicles at different stages of development in normal and experimental monkeys.

Quantitative estimates of the absolute numbers and of the relative numbers of follicles per unit of tissue in the three groups of ovaries have not yet been completed, nor, so far, have the data on nuclear relative to ovular size been analysed statistically. The following conclusions are, however, suggested by a preliminary study of the first of these two questions, and by the facts shown in Table 3.

(1) Treatment of normal monkeys with either oestrogen or androgen reduces the size of the ovary, apparently by suppressing the later stages of follicular development.

(2) The hormones have different, and apparently direct, effects on the earlier stages of growth. Androgen greatly stimulates the early stages of development of the ovum and follicle. In consequence the number of healthy young follicles per unit tissue may become greater than normal. Oestrogen also appears to stimulate the very first phase of differentiation of ova in the ovarian cortex, but it then exercises a suppressive effect on their growth, which is cut short far sooner than would be the case even in surgically hypophysectomized animals.

(3) Only the earlier stages of follicular growth are stimulated by androgen. The relative proportion of ova surrounded by one, two, three and four layers of granulosa cells is higher than in untreated controls. The relative frequency of follicles with an antrum is, however, about the same as in untreated controls. No healthy follicles with an antrum were observed in the two oestrogen-treated animals, although some are present in the ovaries of other rhesus monkeys, not used in the present study, which were under long-term oestrogenic treatment. In the present two animals no healthy follicle was observed with more than three layers of granulosa cells.

#### DISCUSSION

While they lead to certain definite conclusions, the data reported in this study do not provide a full picture of the influence exerted by androgens and oestrogens on the growth of the follicle of the rhesus monkey. Necessary information that has not yet been put together includes, first, estimates of the absolute number of healthy and atretic follicles in the three groups of animals studied; second, corresponding estimates for the relative numbers of follicles per unit volume of ovarian tissue; third, information about the stage at which an antrum develops in the follicle; and fourth, information about the fate of the follicles and ova whose early and healthy development is stimulated by androgen. The available information does, however, allow of certain general observations.

Swezy [1933] has produced evidence which strongly suggests that while the trophic influence of the pituitary is essential for the later stages of follicular growth, the pituitary has an inhibitory effect upon the earliest phases. This conclusion explains the observation that the ovaries of hypophysectomized rats contain many more primordial ova and follicles than do those of normal controls. It is also well known that removal of the pituitary in no way prevents the healthy development of follicles—at any rate as far as the stage of antrum formation. The evidence also suggests, as Pincus [1936] points out, that atresia is the normal fate of all except a relatively small number of medium-sized follicles on which the pituitary gonadotrophins exercise their cyclical influence. The reduction in the weight of the ovary after hypophysectomy is very largely, if not entirely, due to the suppression of the later stages of the follicular cycle.

The fact that both androgens and oestrogens can suppress the gonadotrophic functions of the pituitary, and thereby lead to involution of the gonads, is also thoroughly established [see references in Burrows, 1945]. The evidence detailed in the present paper shows, however, that while the ovaries may become reduced in weight as a result of such 'physiological' hypophysectomy, androgenic and oestrogenic

stimulation have an additional ovarian effect that cannot be explained as an indirect result of some pituitary influence. Androgen stimulates the earlier phases of the follicular cycle, while oestrogen has a suppressive effect.

This conclusion about the influence of androgen on the ovary is to some extent in line with the results of certain studies, summarized by Burrows [1945], that have been made on guinea-pigs, rats and mice—but differs in so far as the treatment in these usually short-term experiments resulted in stimulation not of the earlier, but of the later phases of follicular growth. Unfortunately, none of these investigations has included a detailed quantitative analysis of the phases of the follicular cycle, while others refer mainly to the general ovarian involution that occurs as a result of prolonged androgenic action. It is possible, of course, that the stimulating influence of a short course of androgen on large follicles is an indirect reaction, similar to the luteinizing effect of oestrogen first described by Hohlweg [1934], and mediated by the pituitary.

Corresponding stimulating effects have not been reported as a result of oestrogenic treatment of normal animals and, in general, the many papers on the subject refer only to involution of the ovary or to the stimulation of luteinization, differences in response depending presumably on dosage and the age and phase of the animals studied. Stimulating effects have, however, been reported by Williams [1940, 1944*a, b*, 1945] and others as occurring in hypophysectomized rats. In these experiments it was found that the fall in ovarian weight which occurs after removal of the pituitary can be prevented if a tablet of stilboestrol is implanted at the time of the operation. A decline still occurs in the number of follicles with antra, but the number of follicles with a diameter greater than  $200\mu$  increases. The change is interpreted as indicating a direct effect of oestrogen on the granulosa cells, and it is not elicited if the stilboestrol is given so long after hypophysectomy that the larger follicles have disappeared. As Williams himself points out, oestrogen in these experiments is not acting on primordial follicles, but only on follicles that have reached a much later stage in development, and have retained their 'functional normality'. Pencharz [1940], who has confirmed these observations, states that similar effects cannot be induced in the ovary of the hypophysectomized rat with androgen.

It is difficult to compare these observations on rats with those that we have made on rhesus monkeys, not only because of the species and weight differences, but also because of absolute and relative differences in the duration and intensity of treatment. Nevertheless, it is perfectly clear that the changes described in the present paper are of a different kind. On the one hand, they refer to a much earlier phase of the follicular cycle, and on the other they indicate direct oestrogenic effects that are the reverse of those which would be anticipated if Williams's observations were transferred to the beginning of the follicular cycle. Williams does not yet conclude that a normal physiological mechanism occurs in rats which parallels the changes observed in his experiments, and indeed, if there were, the observations reported in the present paper would make it difficult to suppose that a similar one operated in Primates.

If, in spite of the absence of certain essential data, in particular the absence of evidence about the direct effects of progesterone upon primordial ova and follicles, one attempts to arrange the present observations in the form of a working hypothesis, some conclusions emerge which it would be of interest to test by further experiment.

It is known that the ovaries and adrenal cortex of the female monkey produce both oestrogen and androgen. One might suppose, assuming that androgen has a physiological function in the female, that its influence is predominant during the earlier phase of the menstrual cycle or the start of the follicular cycle, and that it stimulates oogenesis and the early phases of follicular development. As the follicles increase in size, and particularly during the pre-ovulation period of influence of the follicle-stimulating hormone of the pituitary, an increasing production of oestrogen overrides the androgenic influence. In consequence, there occurs an increasing wave of atresia. The oestrogen produced by the cellular elements that comprise the follicle would on this hypothesis itself be the factor responsible for the atretic process which cuts short the development of all except a very few follicles.

The gonadotrophic hormone of the pituitary exercises its effects on only the relatively small number of follicles that take part in the pre-ovulatory burst of follicular growth, and of which all but the one that ovulates in the normal primate cycle also undergo atresia. At the end of this phase of the follicular cycle, the output of oestrogen abruptly falls, and is replaced by a steadily increasing production of progesterone. There is little evidence as yet about the effect of progesterone upon the earlier stages of the follicular cycle, although it is quite clear that its administration is associated with the absence of ripe follicles and corpora lutea. On the basis of the evidence provided by studies of spermatogenesis (see below), we may, however, assume that progesterone does not antagonize the effects of androgen to the same extent as does oestrogen, and that during the fully developed luteal phase of the menstrual cycle, the earlier stages of follicular development are not cut short as they are in the pre-ovulatory part of the cycle when the oestrogenic effect is dominant.

It may be observed that our observations on the effects of androgen and oestrogen on the early stages of the follicular cycle obtain some confirmation from studies of their influence on the seminiferous epithelium. In considering these effects, it should be remembered that oocytes are homologous with primary spermatocytes, and that the extrusion of the first polar body at the time of ovulation is homologous with the division of the primary into secondary spermatocytes. The subsequent division of the secondary oocyte and the first polar body corresponds to the formation of the four spermatids, which differentiate into the four spermatozoa that are derived from a single primary spermatocyte.

Both oestrogens and androgens induce testicular atrophy and a considerable disturbance of the spermatogenic process, the involution usually being regarded as an effect of the suppression of the gonadotrophic functions of the pituitary. The seminiferous tubules in such experiments do not usually manifest any stage of spermatogenesis later than that represented by a few primary spermatocytes [Halpern & D'Amour, 1934, working on rats]. Preliminary observations that we have made on monkeys suggest, however, that the germinal epithelium does not react in the same way to the two treatments. Thus the seminiferous tubules of a rhesus monkey that was injected daily with oestrogen for a period of almost three years appear as thin cords of cells in which it is impossible to differentiate epithelium characteristic of any except the earliest stage of normal spermatogenesis. On the other hand, the involuted testes of a monkey that had been treated for a long period with androgen shows considerable activity of the seminiferous epithelium, in spite of the general involution of the organ.

Recently, Selye & Friedman [1941], and Shay, Gershon-Cohen, Paschkis & Fels [1941] have attempted to differentiate the effects observed in the testes of rats on the basis of the amount of androgenic treatment received by the animals. Both papers maintain that small doses of androgen lead to a marked decrease in the weight of the testes, and inhibition of spermatogenesis. Higher doses lead to an increase in testis weight and to a stimulation of spermatogenesis. In similar experiments, Rubinstein & Kurland [1941] obtained somewhat different results, in so far as they found that above a certain level, high doses of androgen, while they do not impair the proliferation of the germinal epithelium, depress testicular weight.

Androgenic treatment undoubtedly has a stimulating effect on the testes of hypophysectomized animals. Thus if androgen is given to rats at the time of the removal of the pituitary, spermatogenesis, including the formation of motile spermatozoa, continues for a period. If the androgenic treatment is not begun at the same time as the pituitary is removed, and the testes are allowed to involute, the injected androgen will not restore spermatogenesis. Smith [1945] has observed similar effects in the hypophysectomized monkey, but has shown in addition that androgen will exert its effects at any time provided that a few spermatogonia are spared in the process of post-hypophysectomy involution. Hamilton & Leonard [1938] have shown quite clearly that the effect is due to some direct action on the germinal epithelium, while Nelson [1936, 1937, 1938] has demonstrated that it can also be elicited with progesterone, but not with oestrogen.

The evidence is fairly clear, therefore, that androgen, but not oestrogen, has a direct and stimulating effect upon the spermatogenic cycle. The findings reported in the present paper suggest that this effect of androgen is not limited to the male.

#### SUMMARY

1. The regression lines relating the size of ovum and developing follicle have been determined for eight normal rhesus monkeys, two monkeys treated with oestrogen, and three with androgen.

2. No statistically significant differences have been found between the regression coefficient for either phase of follicular growth in the three groups of animals.

3. Regression coefficients in normal monkeys may vary over a wide range. It would seem that it is necessary to determine the relationship between size of ovum and follicle in several animals of a species before assigning to the species definite regression values for this relationship.

4. Oestrogen not only prevents the later stages of follicular development, but also causes widespread atresia in the earlier stages of follicular growth.

5. Androgen considerably stimulates the healthy development of the early stages of follicular growth, but does not affect the later stages.

6. The influence of oestrogens and androgens on the early stages of follicular development appears to constitute a direct effect on the ovaries.

The hormones used in these experiments were provided by Dr Miescher of the Ciba Company.

The animals were obtained with the aid of a grant to S. Z. from the Medical Research Council; the work was also supported by a grant to S. Z. from the Nuffield Medical Committee, Oxford.

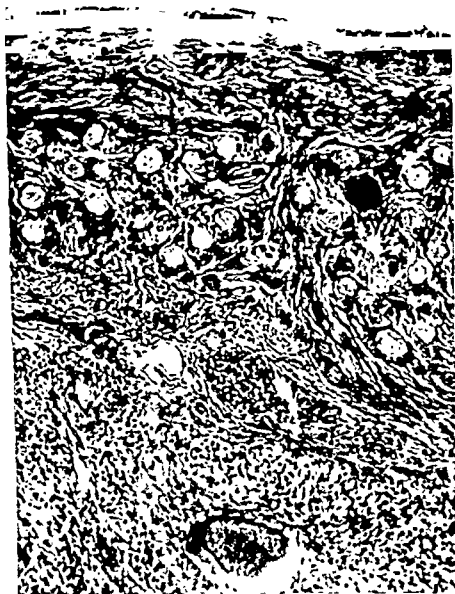


FIG. 4. OM 390. Normal mature monkey.  
× 64.

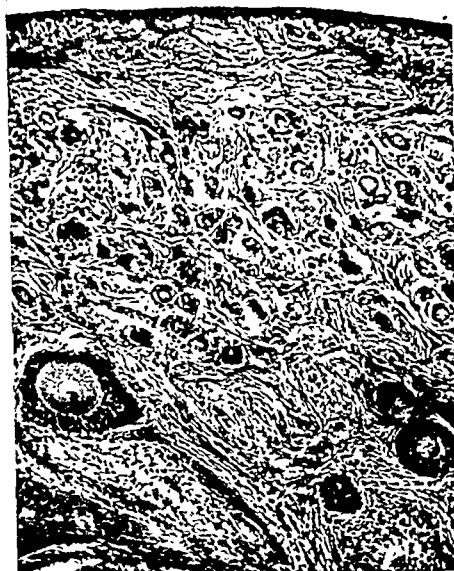


FIG. 5. OM 171. Normal immature monkey.  
× 64.

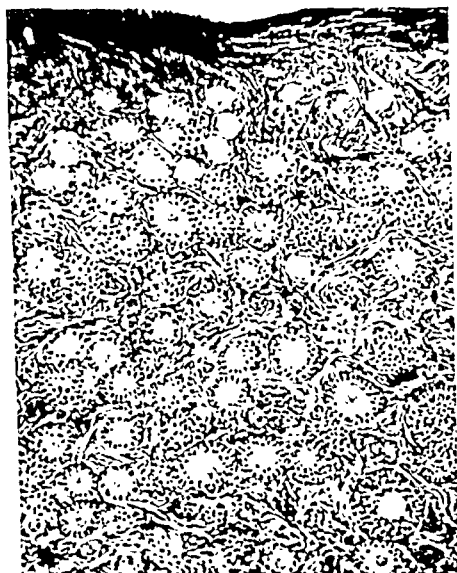


FIG. 6. OM 439. Androgen-treated monkey.  
× 64.

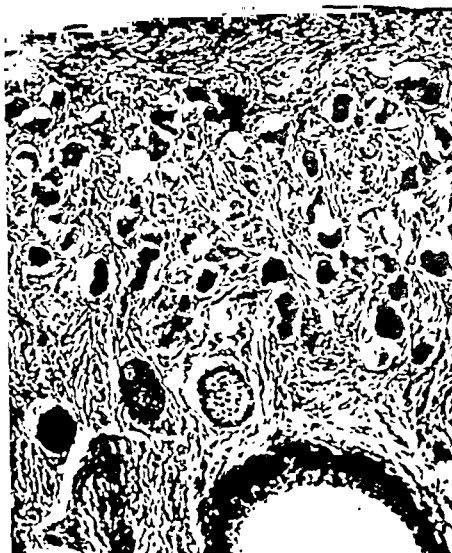


FIG. 7. OM 437. Oestrogen-treated monkey.  
× 64.

Ovarian follicles in normal and treated monkeys.

The four prints are from the cortical zone of the ovary, the germinal epithelium being at the upper edge of the print.





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# DURATION OF REPRODUCTIVE LIFE IN THE BABOON

By S. ZUCKERMAN, *From the Department of Anatomy, Birmingham University*

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Hartman [1938] appears to be the only worker who has recorded information about the duration of reproductive life in subhuman primates. Two rhesus monkeys, *Macaca mulatta*, which he had under observation, died at the ages of 17 and 18, and both continued menstruating almost until the end. Both animals, however, ceased ovulating between their 16th and 17th years.

Although they do not give the full span of activity of the reproductive organs, the following two case histories extend Hartman's observations in so far as they indicate that the interval between puberty and menopause in monkeys may be 20 years or more. The information is being published because inquiries have been made about the possibility that the cessation of menstruation in monkeys under experiment may in some cases be due to old age rather than to experimental interference.

## OBSERVATIONS

### Anubis baboon (*Papio anubis*)

A large female of this species arrived in the London Zoological Gardens on 10 January 1927. She was under observation from 1 March 1929, when she was fully mature [Zuckerman, 1930]. Twenty-five successive cycles were observed between March 1929 and July 1931. They ranged from 30 to 41 days, with an average length of  $34.7 \pm 0.48^*$  days.

The animal was again under observation during 1935. The average duration of four menstrual cycles was 32.5 days, the range of variation being 31–35 days.

Observations were resumed on the same animal in October 1946, and have been continued to the present day (17 June 1947). During this period five phases of menstrual bleeding have been recorded, the average cycle length being 50 days, and the range 39–75 days. It is possible that a phase of uterine bleeding was missed during the one interval of 75 days. Assuming it was, the mean cycle length at present becomes 40 days. Cyclical changes in the sexual skin are not as pronounced at the present time as they were when observations began on this animal.

Since regular menstrual periods were fully established at the beginning of 1930, and since the animal was fully mature physically at the time, it may be safely assumed that her reproductive organs have so far been active for a period of fully 20 years.

### Yellow baboon (*Papio cynocephalus*)

This baboon arrived in the London Zoological Gardens on 30 September 1929, and first manifested changes in the sexual skin at the beginning of 1931. The animal was not fully mature at the time. She was kept under observation for about a year.

\* Standard error of the mean.

During this period regular menstrual cycles, associated with regular changes in the sexual skin, were established. The mean length of eleven cycles observed during this period was  $30.9 \pm 0.95$  days, the range being 25–37 days.

The animal was again under observation during 1935, when six menstrual cycles were followed. Their average length was 27.5 days, and the range 24–29 days.

Observations were resumed on this animal in September 1946, and have been continued up to the present date. During this interval eight periods of menstrual bleeding have been observed, the average periodicity being  $33.3 \pm 1.45$  days, with a range of 27–41 days. The circumgenital skin of this animal no longer swells to the same extent as it did at the beginning of her sexual life, and it is possible that irregularities in cycle length which have been observed during the past year are indicative of the onset of the menopause.

Up to date, the duration of reproductive life in this animal is therefore 17 years.

#### DISCUSSION

The indication that reproductive life in monkeys may last as long as 20 years makes it of interest to compare its duration with that in man. Puberty in the rhesus macaque, *M. mulatta*, usually begins between the 30th and 36th months. Such records as are available suggest that in baboons it begins in the third or fourth year. Information about longevity in these animals is scanty, but it indicates that monkeys may certainly live up to 30 years in captivity. There is one record of a chacma baboon living to 45 years [Flower, 1931].

Corresponding approximate figures for man are: menarche, 12–15 years, with a mean of about 13 years [Ellis, 1947]; menopause 40–50; longevity, say, about 70.

Judging by the stage of dentition, puberty occurs in monkeys and baboons at a relatively younger age than the corresponding human growth phase. Such information as there is suggests, therefore, that the duration of reproductive life in monkeys and baboons relative to the duration of life itself is not less than it is in man, and that it may be longer.

#### SUMMARY

Regular menstrual cycles have occurred in an Anubis baboon, *Papio anubis*, for a period of some 20 years, and in a yellow baboon, *Papio cynocephalus*, for 17 years.

The 1946–7 observations on the two baboons were made by Mr W. Peckit of the London Zoological Society, to whom my best thanks are due.

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# THE PHYSIOLOGICAL ACTIVITY OF THE OPTICALLY ACTIVE ISOMERS OF THYROXINE

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After *dl*-thyroxine had been resolved by Harington in 1928, the activities of the *d*- and *l*-isomers were assayed by Gaddum on tadpoles and rats and on humans by Salter, Lerman & Means. Gaddum [1930] demonstrated that the *l*-isomer is considerably more active than the *d*-form, the ratio of activity varying between 1.5 and 3 according to the dose used. On the other hand, Salter *et al.* [1935], using patients with myxoedema as test objects, found that *d*-thyroxine is as active as *l*-thyroxine. They suggested three conditions that might explain these differences. (1) The animals possessed intact thyroids which might trap newly administered iodine and which might fluctuate in activity. (2) The test material was administered to the animals in single massive doses instead of small daily doses which the patients with myxoedema received. In fact, in Gaddum's experiments, the ratio of activity approached unity as the dose administered became smaller. (3) The difference in species has to be considered.

Some investigators have indicated that *d*-thyroxine has no activity. For example, Foster, Palmer & Leland [1936] found that *l*-thyroxine has twice the metabolic activity of the *dl*-mixture in guinea-pigs, and therefore inferred that the *d*-form has no activity whatever. More recently, Reineke & Turner [1943, 1945], working with *l*-thyroxine isolated from iodinated casein, came to the same conclusion.

Now that newer methods of isolating the isomers of thyroxine are available, the role of optical activity in thyroid physiology can be re-evaluated.

In recent work on the hydrogen peroxide oxidation of *l*(+)-di-iodotyrosine, Harington & Pitt Rivers [1945] have obtained *l*(-)-thyroxine in small yields (up to 4%); there is no reason to suppose that racemization occurs during the oxidation; indeed, the specific rotation of the thyroxine thus obtained was higher than that obtained either by resolution of the *dl*-amino-acid [Harington, 1928] or by isolation from the thyroid gland by Foster *et al.* [1936]. This led to the hope that if *d*(-)-di-iodotyrosine could be prepared in quantity, sufficient *d*(+)-thyroxine might be obtained to test its activity on cases of human myxoedema.

At the time when these experiments were planned, the resolution of *dl*-tyrosine by Sealock [1946] was not available. During the present work, *d*(+)-tyrosine was first prepared by the action of the *l*-amino-acid oxidase of *Proteus vulgaris* [Stumpf & Green, 1944] on *dl*-tyrosine, but the method was cumbersome and did not lend itself to large-scale preparation.

An attempt was therefore made to invert *l*(-)-tyrosine by the action of nitrosyl bromide through the bromo-acid to the *d*(+)-amino-acid; this was successful and has led to the following synthesis. *N*-Carbobenzyl-oxytyrosine ester [Bergmann & Zervas,

1932] was converted into *O*-methyl-*N*-carbobenzyloxytyrosine by the action of dimethyl sulphate and excess of alkali; catalytic reduction led to *O*-methyl-*L*-tyrosine. The latter compound had already been prepared by Behr & Clarke [1932] by the methylation and subsequent hydrolysis of *N*-acetyl-*L*-tyrosine, but this was not done under the conditions defined by Bergmann & Zervas [1928] under which tyrosine must be acetylated in order to avoid any racemization. Behr & Clarke did in fact obtain a partly racemized product after acetylation, and their *O*-methyl-*L*-tyrosine did not have such a high specific rotation as that prepared in the present work where the conditions of acetylation are not conducive to racemization.

*O*-Methyl-*L*-tyrosine was converted by the action of nitrosyl bromide into  $\alpha$ -bromo- $\beta$ -(*p*-methoxy-phenyl) propionic acid; treatment of this acid with ammonia gave a good yield of *O*-methyl-*d*-tyrosine, but at this stage of the synthesis, some racemization did occur. It was not found possible to obtain *O*-methyl-*d*-tyrosine with as high a rotation as the *O*-methyl-*L*-tyrosine, although a fairly optically pure product was obtained by conversion of the bromo-acid to the amino-acid at 0°. The *d*-tyrosine obtained by demethylation of this somewhat racemized *O*-methyl-*d*-tyrosine was easily obtained optically pure by one crystallization from water, since the racemate is more soluble than optically active tyrosine.

Iodination of *d*-tyrosine either by the method of Harington [1928] or by that of Block & Powell [1943] gave good yields of *d*(-)-di-iodotyrosine, and hydrogen peroxide oxidation of the latter led to *d*(+)-thyroxine with a specific rotation equal in magnitude to that of *l*(-)-thyroxine prepared by Harington & Pitt Rivers [1945].

## EXPERIMENTAL

### *Chemical synthesis*

*O*-Methyl-*N*-carbobenzyloxytyrosine. *N*-Carbobenzyloxytyrosine ester [Bergmann & Zervas, 1932] (12.4 g.) was dissolved in *N*-NaOH (88 ml.) and kept for 15 min. at room temperature. The solution was then treated alternately with 5*N*-NaOH (4 ml.) and dimethyl sulphate (2 ml.) with vigorous shaking; the additions were made four times in all, giving 100 % excess of dimethyl sulphate.

At the end of the reaction the solution was acidified with dilute HCl; the oily precipitate solidified on scratching and was recrystallized from aqueous alcohol. It formed fine colourless needles; the yield was 90 % of the theoretical; m.p. 112°. Found: N, 4.24 %;  $C_{18}H_{19}O_5N$  requires N, 4.26 %.

*O*-Methyl-*L*-tyrosine. The preceding compound (11.9 g.) was dissolved in 50 ml. of 60 % alcohol and 7 ml. of 3*N*-HCl and reduced catalytically in the presence of palladium black (1 g.). At the end of the reduction, the solution was filtered and concentrated under diminished pressure. On cooling, the solution deposited 5.5 g. of crystals (m.p. 237–8° decomp.) and by concentrating the mother liquor a second crop of 0.32 g. was obtained with the same melting-point. This is the hydrochloride obtained by Behr & Clarke (m.p. 237–8° decomp.). The acid was obtained by dissolving the hydrochloride in water and neutralizing the hot solution with ammonia; it had m.p. 259–260° (decomp.) ( $\alpha_D^{25}$  = +10.0° in 2 % solution in *N*-HCl).

$\alpha$ -Bromo- $\beta$ -(*p*-methoxyphenyl) propionic acid. *O*-Methyl-*L*-tyrosine (17.0 g.) was dissolved in 3*N*-sulphuric acid (145 ml.) and chilled in ice-salt. Potassium bromide

(35 g.) was added and stirred till dissolved. Sodium nitrite (7.85 g.) in saturated aqueous solution was added with mechanical stirring over a period of 5 hr., the temperature of the reaction mixture being kept between  $-15$  and  $-8^{\circ}$ . At the end of the reaction, a large part of the product had crystallized out. A further quantity was obtained by cautious dilution with water. The product was collected, washed with water and dried *in vacuo*. The yield was 73 % of the theoretical; m.p.  $98-99^{\circ}$ . Found: Br, 29.8 %;  $C_{10}H_{11}O_3Br$  requires Br, 30.9 %. Although this analysis indicates that the product was not quite pure, satisfactory yields of *O*-methyl-*d*-tyrosine were obtained from it.

*O*-Methyl-*d*(+)-tyrosine. The bromo-acid described above (6.8 g.) was dissolved in 100 ml. of ice-cold ammonia (sp.gr. 0.880) and kept at  $0^{\circ}$  for 48 hr.; the solution was then kept at room temperature for a further 48 hr., after which it was concentrated to dryness under diminished pressure; the solid residue was extracted with warm 90 % alcohol, filtered, washed and dried; the yield was 74 % of the theoretical. After crystallization from hydrochloric acid, the hydrochloride had m.p.  $236-8^{\circ}$  and the free amino-acid obtained from it had m.p.  $252^{\circ}$  (decomp.).  $(\alpha)_D^{21} = +8.2^{\circ}$  in 1.85 % solution in *N*-HCl. Recrystallization of the *O*-methyl-*d*-tyrosine from water did not raise the specific rotation. Found: N, 7.2 %;  $C_{10}H_{13}O_3N$  requires N, 7.18 %.

*d*(+)-Tyrosine. *O*-Methyl-*d*-tyrosine (12.8 g.) was boiled under reflux with 7.5 g. of red phosphorus, 24 ml. of acetic anhydride, 50 ml. of acetic acid and 75 ml. of hydriodic acid (sp.gr. 1.7) for  $\frac{1}{2}$  hr. The reaction mixture was filtered hot from the red phosphorus and concentrated to dryness under diminished pressure. The crystalline residue was dissolved in hot water containing a little sodium bisulphite and neutralized with ammonia (pH 5.2); after leaving at  $0^{\circ}$  for 24 hr. the product was collected, washed with water and dried.  $(\alpha)_D^{21} = +10.2^{\circ}$  in 2 % solution in *N*-HCl. It was recrystallized from  $2\frac{1}{2}$  l. of water and gave 6.65 g. (60 %) of *d*-tyrosine with  $(\alpha)_D^{21} = +11.65^{\circ}$  in 2 % solution in *N*-HCl. This rotation agrees with the findings of Stein, Moore & Bergmann [1942], who investigated the temperature coefficient of the specific rotation of *l*-tyrosine.

*d*(-)-Di-iodotyrosine. Iodination of *d*(+)-tyrosine gave *d*(-)-di-iodotyrosine which had  $(\alpha)_D^{25} = -1.6^{\circ}$  in 5 % solution in *N*-HCl.

*d*(+)-Thyroxine. Oxidation of *d*(-)-di-iodotyrosine by the method of Harington & Pitt Rivers [1945] yielded 177 mg. of *d*(+)-thyroxine with  $(\alpha)_D^{25} = +5.15^{\circ}$  at a concentration of 3.3 % in a mixture of 1 volume of *N*-NaOH and 2 volumes of ethanol.

### Clinical trials

The activity of *l*-thyroxine has been assayed in two cases of myxoedema and that of *d*-thyroxine in four cases of myxoedema. The material was injected intravenously daily, sufficient thyroxine having been dissolved to last 1 or 3 days. The results are charted in Figs. 1 and 2.

### RESULTS

One patient, E.D. in Fig. 1, received *d*-thyroxine daily, in dosage containing 0.5 mg. of iodine, for 8 days. The basal metabolism rose from  $-28$  to  $-23$ , but there was no change in weight or in clinical condition. This was considered a negative response. She was then placed on a daily dose of *l*-thyroxine containing 0.25 mg. of iodine. The



metabolic response, as seen in Fig. 2, was of the same order of magnitude as the standard response to 0.5 mg. of iodine in the form of thyroxine polypeptide but at a higher level. The basal metabolism rose to +12, and there was rapid improvement in the myxoedematous symptoms.

The second patient with myxoedema, C.J. in Fig. 1, also failed to show any response to *d*-thyroxine in amounts containing 0.5 mg. of iodine for 9 days, but made a standard response to *l*-thyroxine in a daily dose of 0.25 mg. of iodine (see Fig. 2).

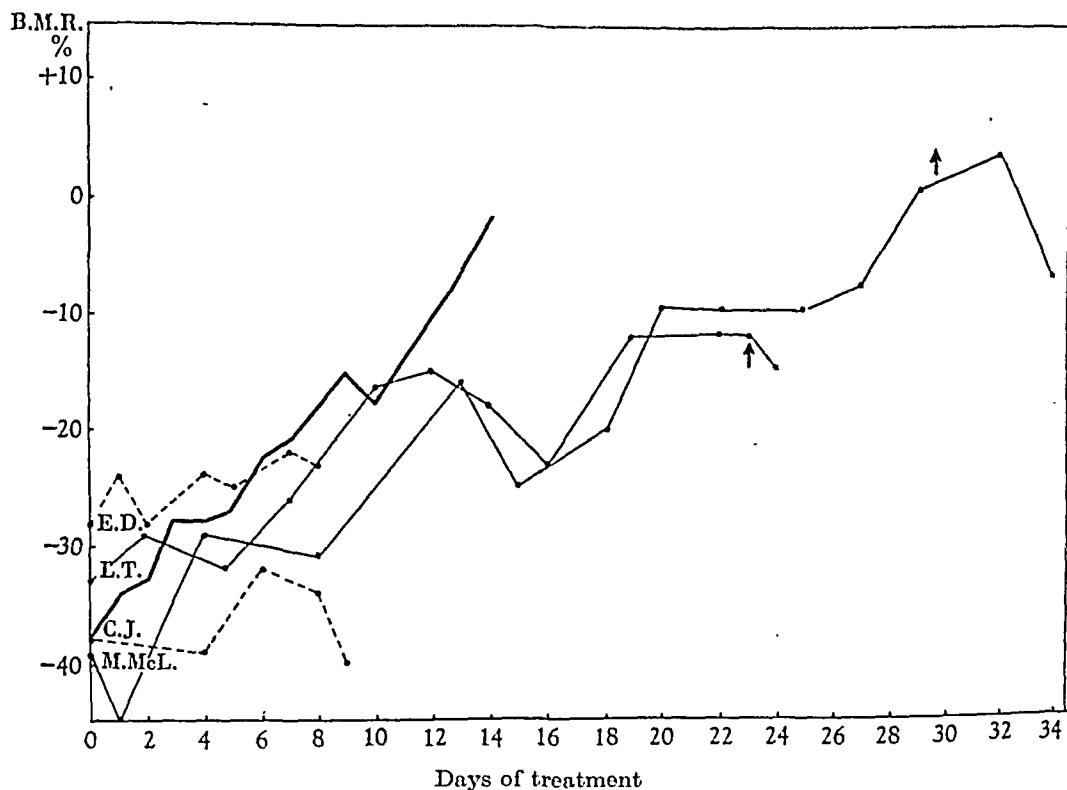


FIG. 1. The calorogenic response of patients with myxoedema to *d*-thyroxine. The interrupted curves represent the response to daily intravenous doses containing 0.5 mg. of iodine. The two light curves represent the response to daily intravenous doses containing 1 mg. of iodine. The heavy line is the standard response to daily doses of 0.5 mg. of iodine in the form of thyroxine polypeptide. The small arrows indicate day of final dose.

The third patient, M.McL. in Fig. 1, a case of juvenile myxoedema, made a substandard response to daily administration of *d*-thyroxine in amounts containing 1 mg. of iodine. At the end of 30 doses, the basal metabolism had risen to about +3.

The fourth patient, L.T. in Fig. 1, made a similar response to *d*-thyroxine containing 1 mg. of iodine. She levelled off at -12 in 24 days. In both of these cases the clinical symptoms improved and the serum cholesterol dropped, in the case of M.McL. from 350 to 131 mg./100 ml. and in the case of L.T. from 344 to 255 mg./100 ml. It would seem, therefore, that *d*-thyroxine possesses physiological activity, but the order of activity is not as great as that of the standard thyroxine polypeptide, or of *l*-thyroxine. The ratio of activity between *d*- and *l*-forms is about 1:8 or 1:10.

## DISCUSSION

It is clear from the above results that *l*-thyroxine is about twice as active as the racemic form. On the other hand, *d*-thyroxine also has calorigenic activity. It does not seem likely that the activity of *d*-thyroxine is due to contamination by *l*-thyroxine or that *d*-thyroxine would undergo racemization *in vivo*. In fact, it would be unusual for the isomer of a naturally occurring, physiologically active compound not to possess some degree of activity.

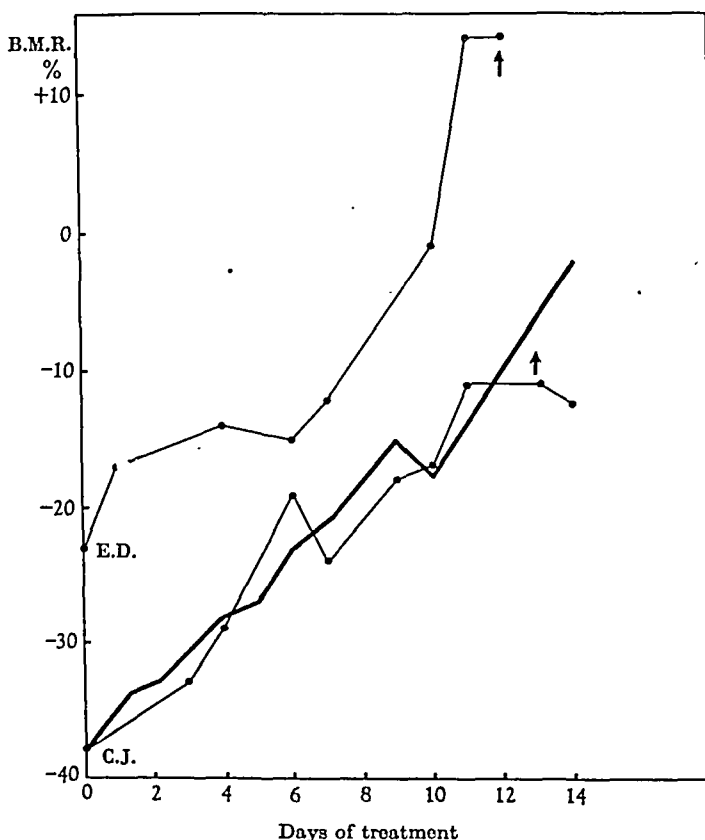


FIG. 2. The calorigenic response of two patients with spontaneous myxoedema (E.D. and C.J.) to daily intravenous doses of *l*-thyroxine containing 0.25 mg. of iodine, shown in light lines. The heavy curve is the standard response to daily doses of 0.5 mg. of iodine in the form of thyroxine polypeptide. Small arrows indicate day of final dose.

The question whether the activity of the whole thyroid is due to its total iodine content or its thyroxine content has been debated for some time, and is not yet resolved. It is possible that the high activity of the whole gland is accounted for by the high activity of *l*-thyroxine as compared with an equivalent amount of the *d*-amino-acid, but further work is required to settle this point.

## SUMMARY

*d*-Thyroxine has been prepared, and its physiological activity in myxoedematous patients has been found to be between one-eighth and one-tenth that of *l*-thyroxine.

We wish to thank Dr C. R. Harington and Dr J. H. Means for their constant interest and encouragement during this work; we also wish to thank Dr Harington for the suggestion that we might be able to prepare *d*-tyrosine by the inversion method.

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# ALLOXAN-DIABETES AND LIVER GLYCOGEN

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A decrease in the glycogen content of the diabetic liver is often considered to be the direct result of the diabetic disturbance in metabolism, caused either by an increased glycogenolysis or by a decreased ability to synthesize glycogen. In previous experiments [Tuerkischer & Wertheimer, 1946] it was observed that the livers of alloxan-diabetic rats and of pair-fed, normal rats contained equal amounts of glycogen. The liver glycogen of alloxan-diabetic rats starved for 24 hr. was actually considerably higher than that of normal, control rats, a fact also reported shortly thereafter by Weber [1946]. Additional burdening of the carbohydrate metabolism with complete exhaustion of the carbohydrate reserves (by phloridzin or swimming) caused increased glycogenesis in the diabetic rat, starved for 24 hr., with an increased deposition of glycogen in the liver. This increase in liver glycogen was only found in non-comatose, alloxan-diabetic rats. It was therefore assumed that the decrease in the glycogen content of the diabetic liver is not due to the diabetes *per se*, but that an additional lesion exists in acidotic rats which, together with the diabetes, induces either an increase in glycogenolysis or a decrease in the synthesis of glycogen.

In further investigations based on these results the following experiments were carried out.

(1) In addition to the experiments described in 1946 the liver glycogen of starved, non-acidotic, diabetic rats was investigated following (a) casein-rich, and (b) fat-rich dieting.

The response of the liver glycogen to insulin in non-comatose, and in comatose, diabetic rats was compared.

(2) Experiments were carried out with non-comatose, and comatose, rats on the formation of liver- and muscle-glycogen following the administration of various sugars and other glycogen-forming substances with or without insulin.

(3) The influence of liver injury on the production of acidosis in diabetes was investigated.

## METHODS

Young male albino rats of laboratory stock, weighing 90–130 g., were used. Diabetes was produced by the intravenous injection of 6–8 mg. of alloxan. The animals were used for experiment when glycosuria was established for at least 2 days. The rats were usually maintained on a diet of wheat and vegetables. In addition the following diets were used.

- (1) Carbohydrate-rich diet (70 % carbohydrate, 20 % casein, 10 % fat).
- (2) Casein-rich diet (70 % casein, 20 % carbohydrate, 10 % fat).
- (3) Fat-rich, protein-poor diet (45 % fat, 50 % carbohydrate, 5 % casein).
- (4) Fat-rich, protein-rich diet (45 % fat, 50 % casein, 5 % carbohydrate).
- (5) Carbohydrate-rich, protein-free diet (90 % carbohydrate, 10 % fat).
- (6) Protein-rich, carbohydrate-poor diet (80 % casein, 10 % carbohydrate, 10 % fat), or alternatively a pure meat diet.

All these diets were supplemented with the usual additions of minerals and vitamins.

Carbon tetrachloride was injected according to the method of Cameron & Karunaratne [1936] twice weekly in doses of 0.1 ml./100 g. body weight.

The various sugars and other glycogen-forming substances were given by stomach tube. The insulin used was Insulin Squibb.

The animals were killed under Nembutal narcosis. Glycogen was determined according to a modification of the method of Good, Kramer & Somogyi [1933]. Glucose was estimated according to the method of Shaffer, Hartmann & Somogyi [Somogyi, 1937], acetone according to that of Weichselbaum & Somogyi [1941] and urea according to that of Conway [1940]. Liver fat was estimated as described by Schiffer & Wertheimer [1947].

## RESULTS

### *The formation of glycogen in comatose or non-comatose alloxan-diabetic rats*

The increased deposition of liver glycogen previously described after 24 hr. starvation in diabetic rats on a diet of wheat or on a synthetic high-carbohydrate diet was also observed in rats which were kept for about 2 weeks on a casein-rich, or a fat-rich, diet before the fast. The results of these experiments are given in Table 1.

Table 1. *The liver and muscle glycogen of alloxan-diabetic and normal rats after starvation*

Diet	Animals	No. of exps.	Glycogen (g./100 g.)		Blood sugar (mg./100 ml.)
			Liver	Muscle	
Casein-rich	Diabetic	16	$1.13 \pm 0.16$	$0.22 \pm 0.02$	$235 \pm 44$
	Control	12	$0.32 \pm 0.09$	$0.20 \pm 0.02$	—
Fat-rich	Diabetic	7	$1.30 \pm 0.23$	$0.29 \pm 0.008$	$223 \pm 29$
	Control	9	$0.63 \pm 0.11$	$0.28 \pm 0.07$	—

Administration of two doses of 1 mg. of a dead and washed preparation of *Salmonella typhi* murium 'endotoxin' suppressed the deposition of glycogen in the livers of starved, diabetic rats. Nor was there any increase in liver glycogen in starved, diabetic rats which were exposed to cold for 6 hr. at the end of a 24–34 hr. fast (Table 2).

Table 2. *The liver glycogen of alloxan-diabetic and normal rats during fasting in conjunction with administration of 'endotoxin' or with exposure to cold*

Treatment	Animals	No. of rats	Glycogen (g./100 g.)		Blood sugar (mg./100 ml.)
			Liver	Muscle	
'Endotoxin'	Diabetic	6	$0.05 \pm 0.004$	$0.12 \pm 0.04$	$230 \pm 35$
	Control	4	$0.05 \pm 0.012$	$0.10 \pm 0.01$	—
6 hr. exposure to cold	Diabetic	14	$0.09 \pm 0.015$	$0.18 \pm 0.017$	$235 \pm 92$
	Control	14	$0.14 \pm 0.025$	$0.20 \pm 0.018$	—

A small increase in the liver glycogen of diabetic rats was found, however, when after exposure to cold, the animals were allowed to recover for 4–5 hr., liver glycogen values being  $0.24 \pm 0.08$  g./100 ml. for the diabetic and  $0.08 \pm 0.02$  for the normal animals (17 expts.).

In comatose, alloxan-diabetic rats, characterized by ketonuria and ketonaemia, decrease in body temperature, drowsiness, and fatty liver, no increase in the liver glycogen was observed during fasting. Although in a few cases ketonuria was absent, marked ketonaemia indicated the existing acidosis (see Table 3).

Administration of two doses of 0.3 units of insulin to non-comatose and to comatose diabetic rats during the last 4 hr. of the 24 hr. starvation period had different results in these two groups of rats, as may be seen in Table 3.

Table 3. *The liver and muscle glycogen of non-acidotic and acidotic, alloxan-diabetic rats after 24 hr. starvation and the response to insulin administered at the end of the starvation period*

		No. of rats	Glycogen (g./100 g.)		Blood sugar (mg./100 ml.)
Treatment	Animals		Liver	Muscle	
Without insulin					
24 hr. fast	Non-acidotic	34	0.70 ± 0.10	0.23 ± 0.02	299 ± 36
	Acidotic	20	0.13 ± 0.05	0.23 ± 0.03	513 ± 73
Without fast	Acidotic	12	0.17 ± 0.07	0.25 ± 0.03	616 ± 84
With insulin					
24 hr. fast	Non-acidotic	11	0.11 ± 0.03	0.18 ± 0.03	66 ± 17
	Acidotic	7	0.85 ± 0.23	0.11 ± 0.02	180 ± 55

*The formation of liver glycogen following the administration of various sugars and other glycogen-forming substances*

The test animals were starved for 20–24 hr. before the experiment. The volume of liquid administered by stomach tube was 2–2.5 ml./100 g. body weight. The results of these experiments are summarized in Table 4.

Table 4. *The liver and muscle glycogen of alloxan-diabetic and normal rats following the administration of various glycogen-forming substances*

Substance	Dose (mg./100 g.)	Animals	Killed after hr.	No. of rats	Glycogen (g./100 g.)		Blood sugar (mg./100 ml.)
					Liver	Muscle	
Glucose	625	Diabetic	2–4½	11	$2.76 \pm 0.46$	$0.29 \pm 0.025$	$389 \pm 85$
		Control	2–4½	11	$1.30 \pm 0.26$	$0.44 \pm 0.04$	$127 \pm 5$
		Diabetic	6	9	$1.30 \pm 0.23$	$0.20 \pm 0.026$	$300 \pm 81$
		Control	6	8	$0.79 \pm 0.21$	$0.28 \pm 0.026$	$112 \pm 8$
Fructose	625	Diabetic	3–4	5	$1.37 \pm 0.28$	$0.23 \pm 0.024$	$349 \pm 62$
		Control	3–4	5	$1.69 \pm 0.16$	$0.23 \pm 0.013$	$127 \pm 4$
		Diabetic	6	8	$2.02 \pm 0.20$	$0.23 \pm 0.037$	$195 \pm 26$
		Control	6	10	$1.34 \pm 0.24$	$0.30 \pm 0.039$	$106 \pm 8$
Galactose	625	Diabetic	3–6	6	$1.10 \pm 0.26$	$0.20 \pm 0.010$	$287 \pm 79$
		Control	3–6	6	$0.71 \pm 0.19$	$0.21 \pm 0.020$	$145 \pm 20$
Succinic acid	150	Diabetic	6–9	11	$0.95 \pm 0.22$	$0.20 \pm 0.02$	$273 \pm 91$
		Control	6–9	13	$0.47 \pm 0.09$	$0.25 \pm 0.02$	$95 \pm 7$
Lactic acid	400	Diabetic	5–7	10	$0.74 \pm 0.23$	$0.12 \pm 0.021$	$169 \pm 22$
		Control	5–7	9	$0.93 \pm 0.33$	$0.27 \pm 0.030$	$100 \pm 8$
Alanine	360	Diabetic	6–7	6	$0.59 \pm 0.37$	$0.20 \pm 0.034$	$212 \pm 40$
		Control	6–7	7	$0.62 \pm 0.15$	$0.23 \pm 0.034$	$91 \pm 6$
Glycerol	800	Diabetic	3½	7	$1.42 \pm 0.36$	$0.20 \pm 0.040$	$277 \pm 65$
		Control	3½	7	$1.80 \pm 0.29$	$0.28 \pm 0.021$	$149 \pm 8$
		Diabetic	4½–6	10	$1.30 \pm 0.26$	$0.26 \pm 0.020$	$338 \pm 40$
		Control	4½–6	9	$3.38 \pm 0.50$	$0.40 \pm 0.041$	$166 \pm 15$

Administration of glucose or fructose produced no deposition of glycogen in the liver of acidotic, diabetic rats (7 exps.).

The liver glycogen values of diabetic animals which received 0.3 units of insulin simultaneously with the administration of glucose were markedly lower than those of similar animals without insulin treatment ( $0.83 \pm 0.11$  g./100 ml. in 6 exps.).

The liver glycogen of diabetic animals which received 0.3 units of insulin simultaneously with the administration of glycerol was distinctly higher than that of similarly treated normal rats ( $1.37 \pm 0.39$  for the diabetic as compared with  $0.75 \pm 0.19$  g./100 ml. for the normal animals; 6 exps.).

*The influence of liver injury on the development of diabetic ketosis*

Rats were kept on a fat-rich, protein-poor diet, this diet being suitable to produce pathological changes such as fatty infiltration, necrosis and even cirrhosis of the liver. The influence of a fat-rich diet on the development of diabetes was recently studied by Houssay & Martinez [1947]. Other rats were kept on a protein-free diet but received injections of carbon tetrachloride. After 2-3 weeks the rats were injected with alloxan. The results of these experiments are listed in Table 5.

Table 5. *The influence of the diet and of liver injury on the development of alloxan-diabetes*

Diet	No. of rats	Percentage of cases with			Liver fat (g./100 g.)	Blood sugar (mg./100 ml.)	Blood acetone (mg./100 ml.)	Blood urea (mg./100 ml.)
		Acetone-uria	Fall of body temp.	Liver-glycogen (g./100 g.)				
Fat-rich, protein-poor	12	92	67	$0.28 \pm 0.14$	$10.8 \pm 1.2$	$541 \pm 74$	101*	154†
Fat-rich, protein-poor + 5 mg. choline daily	5	100	100	$0.08 \pm 0.006$	$6.6 \pm 0.8$	$550 \pm 83$	$157 \pm 36$	$120 \pm 50$
Protein-rich (Diet 6).	11	73	45	$0.67 \pm 0.15$	$5.7 \pm 0.58$	$383 \pm 87$	$113 \pm 37$	$385 \pm 71$
Carbohydrate-rich, protein-free	9	55	0	$1.68 \pm 0.47$	$6.6 \pm 0.95$	$268 \pm 54$	$12 \pm 3$	$38 \pm 10$
Protein-free + CCl <sub>4</sub> only before alloxan administration	6	50	66	$0.46 \pm 0.13$	$5.1 \pm 0.87$	$618 \pm 48$	$128 \pm 20$	$185 \pm 28$
Protein-free + CCl <sub>4</sub> also after alloxan administration	10	90	70	$0.07 \pm 0.02$	$7.4 \pm 0.84$	$378 \pm 60$	$149 \pm 28$	$198 \pm 23$

\* As the acetone excretion in the urine of these animals was very marked, blood acetone bodies were determined only in two cases.

† Blood urea determined only in two cases.

Diabetic rats maintained on a fat-rich, protein-rich diet were in a better general condition than those maintained on the fat-rich, protein-poor diet. The liver glycogen values were higher, the values for liver fat and for blood sugar lower than those of the latter. Feeding a protein-rich diet was accompanied by extremely high values for blood urea. On the other hand it should be noted that high urea values (as high as 120 mg./100 ml.) were also observed in normal rats, kept on this protein-rich diet (unpublished data).

#### DISCUSSION

Our present experiments, together with those already published in 1946, yielded values for the liver glycogen of a total of 91 diabetic and of 116 normal rats after a 24 hr. fast under different nutritional conditions. The mean liver glycogen obtained

in these experiments was  $1.08 \pm 0.08$  g./100 ml. in the diabetic rats as compared with  $0.29 \pm 0.01$  in the normal animals. These experiments show clearly that the liver glycogen during fasting is increased in the diabetic rats. The results of experiments on recovery after swimming or after exposure to cold during starvation support the assumption that glycogenesis during starvation is enhanced in the diabetic rat. Starvation experiments have also been carried out in rats on a casein-rich or a fat-rich diet. These diets lead to increased glycogenesis after fasting even in the normal rat [Mirski, Rosenbaum, Stein & Wertheimer, 1938; Stein, Tuerkischer & Wertheimer, 1939]. Here the differences between normal and diabetic rats after fasting were somewhat smaller. Exposure to cold or administration of a dead and washed preparation of *Salmonella typhi murium* 'endotoxin' during starvation abolishes the increased glycogenesis.

The findings of Stetten & Böxer [1944] that the formation of glycogen from glucose is decreased in the diabetic rat, but that the formation from 3-carbon-chain breakdown products is increased, serve as a possible explanation for the increased liver glycogen in diabetic rats. As already suggested by Peters [1945], it can be assumed that the formation of blood sugar from these breakdown products proceeds via liver glycogen.

As also reported by Weber [1946], the deposition of liver glycogen following the administration of glucose is markedly enhanced in the diabetic rat. The muscle glycogen of these rats, however, was lower than that of the normal controls. No marked differences in the liver glycogen of normal and alloxan-diabetic rats were found following the administration of fructose, galactose, succinic acid, lactic acid and alanine. Administration of glycerol alone produced remarkable deposition of liver glycogen only in the normal rat, whereas no such increase could be observed in the diabetic animal. No explanation can be given for these findings.

Our results indicate that the synthesis of glycogen in the diabetic rat is not decreased, but under certain conditions is even enhanced. It is proved that formation of liver glycogen from certain precursors is impaired in the diabetic rat, the total amount of glycogen formed, however, being doubtless increased in the diabetic animal. Even when glycogenolysis is enhanced the proportion of glycogenolysis to the existing increased synthesis is such that more glycogen is deposited in the liver of the diabetic than of the normal animal. In the diabetic muscle, however, glycogenolysis is favoured.

These findings do not hold for the comatose, diabetic rat, in which the liver glycogen was always low, even under conditions which caused increased formation of liver glycogen in the non-comatose, diabetic animal. It is assumed that in this case diabetes aggravated by certain liver injury changes the proportion between glycogenolysis and glycogen synthesis in favour of the former. Depletion of liver glycogen is not the expression of the diabetes *per se*, but of the diabetic coma. The injection of alloxan following a lesion of the liver induced by a long period on a fat-rich, protein-poor diet or on a protein-free diet with additional carbon tetrachloride poisoning always produced diabetic coma. The effect of the fat-rich, protein-poor diet could not be abolished by addition of choline. The inhibition of the increased glycogenesis during fast by 'endotoxin' may also be a result of a lesion of the liver.

The widely held view that insulin always increases the glycogen content of the diabetic liver was not supported by our experiments. Only in diabetic coma did



insulin produce noticeable deposition of liver glycogen. In non-comatose, diabetic rats the relatively high glycogen found after starvation fell after insulin administration, as did glycogen deposition from administered glucose. These experiments are in good accord with the *in vitro* experiments of Sutherland & Cori [1947], who found that the action of insulin on liver slices incubated with Cori-ester may be glycogenolytic as well as favouring synthesis, depending on the condition of the liver. Shipley & Humel [1945] also observed the favouring of glycogen synthesis in liver slices of starved animals and the favouring of glycogenolysis in liver slices from well-fed rats.

Another explanation of our experiments should however be considered. It may be possible that the pancreas of alloxan-diabetic rats is still able to furnish enough insulin to allow the homeostatic mechanism of the liver to function at a diabetic blood-sugar level and that only in the acidotic animal is the insulin mechanism completely destroyed and thus diabetes complete. In the non-acidotic, partially diabetic animal, food might temporarily exhaust the scanty insulin supply in the islets. Liver glycogen would be low and blood sugar high in consequence. During starvation the islets then may begin again to secrete small amounts of insulin, blood sugar be reduced and liver glycogen rise. In other words, endogenous insulin from the islets does here what injected insulin does in the acidotic rat. In the partially diabetic rat the lowering of the liver glycogen by insulin would thus be in good accord with the effect of insulin in normal animals, while only in totally diabetic rats does insulin raise the liver glycogen.

The possibility that alloxan might produce partial as well as total diabetes is, however, not in accordance with the following findings. With our relatively small dose of alloxan a certain percentage of the animals always developed coma (i.e. total diabetes). Use of higher doses did not increase the percentage of acidotic animals. A partial diabetes, produced by injection of alloxan, could not be changed by a second injection to total diabetes.

In any case it follows that glycogen formation as a whole is not impaired in the non-acidotic ('partially') diabetic rat and that insulin increases liver glycogen only in the acidotic ('totally') alloxan-diabetic animal.

#### SUMMARY

1. Alloxan-diabetic rats after 24 hr. starvation exhibited markedly increased liver glycogen as compared with normal animals. This effect was independent of the diet. The same low liver glycogen values were found in alloxan-diabetic and normal rats after exposure to cold or after administration of *Salmonella typhi murium* 'endotoxin' during starvation.

2. The deposition of liver glycogen after the administration of various sugars or other glycogen-forming substances by stomach tube was certainly no lower in the non-acidotic, alloxan-diabetic animal. Feeding of glucose even led to a much more distinct deposition in the alloxan-diabetic rats. Of all substances investigated only glycerol caused no distinct glycogen deposition in the liver of the alloxan-diabetic animal as compared with normal animals. The muscle glycogen of alloxan-diabetic rats, however, was low after starvation and no increase was observed after administration of glucose.

3. In comatose, alloxan-diabetic rats, however, no increase in liver glycogen during fast occurred and no deposition of glycogen could be induced in response to the administration of glucose.

4. Administration of insulin during starvation caused an increase in liver glycogen only in alloxan-diabetic, acidotic rats. In non-acidotic rats the liver glycogen decreased after insulin.

5. Injection of alloxan following the production of hepatic lesions by a fat-rich, protein-poor diet or by a protein-free diet with the additional injection of carbon tetrachloride always resulted in diabetic coma with increased acetone-body formation, decrease in body temperature, extreme decrease in liver glycogen and fatty infiltration of the liver.

6. The possible mechanism of the increased glycogen deposition in the liver of alloxan-diabetic animals, and the role of the liver in diabetic coma are discussed.

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# ADRENALECTOMY AND REPLACEMENT THERAPY IN LACTATING RATS

## 4. EFFECT OF DEOXYCORTICOSTERONE ACETATE ON THE WATER CONTENT OF MAMMARY TISSUE AFTER ADRENALECTOMY

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It has been known for many years that milk secretion markedly decreases after adrenalectomy [see Cowie & Folley, 1947*a*]. Recent work indicates that the decrease in milk yield in the rat is due partly to metabolic effects of anorexia resulting from the operation, and partly to more direct effects of the loss of the adrenal hormones [Cowie & Folley, 1947*b*]. Folley & Cowie [1944] and Cowie & Folley [1947*a*] were able partially to restore lactation after adrenalectomy in their rats by administration of deoxycorticosterone acetate (doca). The mammary glands of such treated animals were noticeably heavier even than those of normal sham-operated controls which of course were lactating somewhat better. Since doca will cause growth of the mammary duct and alveolar systems [Van Heuverswyn, Folley & Gardner, 1939; Speert, 1940; Nelson, Gaunt & Schweizer, 1943; Mixner & Turner, 1942], it seemed possible that the increased weight of these glands above normal might be due to new mammary growth superimposed on substantial maintenance of the functional (and presumably therefore structural) *status quo*. An alternative cause for this rather unexpected weight increment might be increased hydration of the mammary tissues. The results described in this paper show that the second explanation is correct.

### EXPERIMENTAL

#### *Animals and treatment*

Hooded Norway rats undergoing their first lactation were used. In some experiments the diet was as described previously [Folley & Cowie, 1944]; in others the provision of fresh milk *ad lib.* was replaced by the incorporation of appropriate proportions of dried milk into the diet [e.g. Cowie & Folley, 1947*c*]. Some groups received a diet containing increased proportions of sodium [Cowie, Folley, French & Greenbaum, 1947] or protein [Cowie & Folley, 1947*c*]. Save in one doubtful case (see below) there is no evidence that any of these dietary changes, which were made for another purpose, had any significance in the present connexion.

Adrenalectomy or, in the case of the controls, a sham operation was performed on the 4th day of lactation, and subcutaneous injections of doca in oil or, where appropriate (controls), of oil alone were thereafter given daily until the 16th day in Exps. 3-5 and until the 20th day in Exps. 1 and 2.

At autopsy (on the day following the last injection) the six abdominal\* mammary glands were dissected off as rapidly, completely, and accurately as possible, and at once weighed on an air-damped balance.

#### *Milk content of mammary tissue*

The approximate amount of milk retained in the mammary alveoli at autopsy was determined from the lactose content of mammary gland homogenates as before [Folley & Greenbaum, 1947], using for sham-operated controls the mean value for the lactose content of normal rats' milk then obtained. In the case of adrenalectomized rats receiving 3.0 mg. of doca daily, it was necessary also to determine the lactose content of their milk. Accordingly in Exp. 5, five such rats were set aside for milking (for technique see Folley & Greenbaum [1947]) and, as was the practice throughout this work, were not used for any other purpose. The mean lactose content for these five milk samples was  $3.33 \pm 0.06$  g./100 ml., a value not very different from that obtained for normal rats' milk [Folley & Greenbaum, 1947]. This value was used, not only for the data of Exp. 5, but also for application to earlier data (Exps. 3 and 4). It was not feasible to obtain milk from untreated adrenalectomized rats and for these the value for control rats was perforce used (but see below).

In this paper the weight of the moist mammary gland after deduction of the weight attributable to the retained milk is referred to as the 'net' mammary gland weight; the 'gross' mammary gland weight being the weight of the moist tissue before application of this correction.

#### *Dry-matter content of mammary tissue*

The dry-matter content of mammary gland tissue and its retained milk was determined (Exp. 5) by weighing a suitable amount of the freshly removed tissue in a small tared beaker containing a little dry sand and a small glass rod. The tissue was dried to constant weight at 100°, this process being hastened by periodic trituration of the tissues with the sand by means of the rod.

In order to calculate from the values thus obtained the true dry-matter content of the mammary tissue, not only the milk content of the tissue, but also the dry-matter content of the milk must be known. In Exp. 5 samples of milk from the five doca-treated adrenalectomized rats specially earmarked for milking, and from three sham-operated controls, were dried at 100° and gave mean values of  $22.17 \pm 0.46$  and  $25.60 \pm 1.38$  g. of dry matter per 100 ml. respectively. The difference is probably significant ( $t=0.977$ ,  $P=0.046$ ). Our values for the lactose and dry matter are similar to those reported by Cox Jnr. & Mueller [1937].

### RESULTS

The results of a number of experiments summarized in Table 1 show that not only the gross weight, but also the net weight of the six abdominal mammary glands was significantly greater in adrenalectomized lactating rats receiving 3.0 mg. of doca daily than in normally lactating controls. The glands from rats receiving a smaller daily dose of doca (0.56 mg.) were intermediate in weight. On the other hand, the glands of

\* For brevity, the two abdominal and four inguinal mammary glands are throughout this paper referred to as the abdominal mammary glands.

Table 1. *Effect of deoxycorticosterone acetate (doca) on the moist weight of the six abdominal mammary glands in the adrenalectomized lactating rat*

Exp. No.	No. of rats	Treatment	Uncorrected (gross) moist weight of abdominal mammary glands (g.)			True (net) moist weight of abdominal mammary glands* (g.)			Remarks
			Mean	S.E.M.	Range	Mean	S.E.M.	Range	
	10	Sham operation	6.63	± 0.50	4.51-10.22	—	—	—	—
	11	Adrx.†	1.86	± 0.22	1.00-3.03	—	—	—	—
	10	Adrx.; 3.0 mg. doca daily	8.80	± 0.65	5.97-12.20	—	—	—	—
	6	Sham operation	6.49	± 0.38	5.49-7.75	—	—	—	High protein diet
	6	Adrx.	1.78	± 0.41	0.00-2.67	—	—	—	High protein diet
	6	Adrx.; 3.0 mg. doca daily	8.87	± 0.92	6.22-11.52	—	—	—	High protein diet
	6	Sham operation	9.52	± 0.82	6.82-12.45	4.41 (a)	± 0.39	3.54-6.26	—
	6	Adrx.	4.03	± 0.60	2.37-6.60	2.77	± 0.42	1.50-4.43	—
	6	Adrx.; 0.56 mg. doca daily	8.37	± 0.87	6.53-11.19	5.07	± 0.30	3.89-5.77	—
	6	Adrx.; 3.0 mg. doca daily	10.79	± 0.71	8.84-13.45	6.13 (b)	± 0.34	5.08-7.65	—
	6	Sham operation	9.49	± 0.68	7.88-11.87	4.06 (c)	± 0.22	3.22-4.69	High sodium diet
	6	Adrx.	5.13	± 0.30	4.51-6.46	3.14	± 0.24	2.55-3.98	High sodium diet
	6	Adrx.; 0.56 mg. doca daily	8.28	± 0.60	6.18-9.97	4.91	± 0.30	4.01-5.77	High sodium diet
	6	Adrx.; 3.0 mg. doca daily	11.48	± 0.96	8.73-13.68	6.58 (d)	± 0.46	4.66-7.84	High sodium diet
	8	Sham operation	8.13	± 0.45	5.76-9.68	4.04 (e)	± 0.28	2.41-4.79	—
	15	Adrx.	4.27	± 0.28	1.79-5.54	2.30	± 0.18	0.75-3.69	—
	7	Adrx.; 0.56 mg. doca daily	7.26	± 0.50	5.80-9.32	4.55	± 0.32	3.73-5.56	—
	6	Adrx.; 0.56 mg. doca daily	7.45	± 0.36	6.40-8.80	4.36	± 0.40	2.43-5.09	High sodium diet
	5	Adrx.; 3.0 mg. doca daily	9.63	± 0.60	7.66-10.92	5.32 (f)	± 0.37	4.77-6.20	—

\* i.e. after deducting the weight of the milk retained in the alveoli and fine ducts.

† Adrx.=adrenalectomy.

For the comparison *a* against *b*,  $t=0.9960$ ,  $P=0.008$ .

For the comparison *c* against *d*,  $t=0.9998$ ,  $P=0.0004$ .

For the comparison *e* against *f*,  $t=0.9912$ ,  $P=0.0176$ .

Table 2. *Percentage water and total dry matter in the six abdominal mammary glands of adrenalectomized lactating rats receiving deoxycorticosterone acetate (doca) and of control lactating rats*

Exp. No.	No. of rats	Treatment	Dry weight of abdominal mammary tissue (g.)		Water content of mammary tissue (%)		Remarks
			Range	Mean	Range	Mean	
	3	Sham operation	1.06-1.88	1.34 (a)	68.6-70.1	69.5	—
	6	Adrx.* 3.0 mg. doca daily	1.06-1.73	1.25 (b)	75.1-77.7	76.9	—
	4	Sham operation	1.05-1.34	1.22 (c)	68.0-72.7	70.3	High sodium diet
	6	Adrx.; 3.0 mg. doca daily	1.10-1.84	1.49 (d)	77.1-77.5	77.3	High sodium diet
	5	Sham operation	0.97-1.78	1.24 (e)	62.0-73.6	69.8	—
	5	Adrx.; 3.0 mg. doca daily	0.90-1.49	1.22 (f)	74.2-81.2	77.2	—

\* Adrx.=adrenalectomy.

For the comparison *b+d+f* against *a+c+e*,  $t=0.8885$ ,  $P=0.223$ .

For the comparison *a* against *b*,  $t=0.7477$ ,  $P=0.505$ .

For the comparison *c* against *d*,  $t=0.9813$ ,  $P=0.037$ .

For the comparison *e* against *f*,  $t=0.5681$ ,  $P=0.864$ .

untreated adrenalectomized rats, the weights of which are given for comparison, were very much lighter than either those of controls or of adrenalectomized rats receiving either dose of doca.

Table 2 gives a summary of the results of direct determinations of the total dry weight of the six abdominal mammary glands, corrected for the dry matter of the milk retained in the tissue, which were carried out in Exp. 5. It will be seen that there was no difference between the dry weights of the glands from sham-operated controls and from doca-treated adrenalectomized rats, indicating that the observed differences in the moist gland weights were wholly due to differences in water content. The values for the water content of the glands from the two groups of rats are given in the last column of Table 2 and show that the glands from the doca-treated adrenalectomized rats contained about 10.6 % more water than those of the sham-operated controls, the absolute difference amounting to 7.4 units of percentage content.

Also included in the table are similar calculations in respect of Exps. 3 and 4, but these are to some extent indirect and must be regarded as supplementary, since to make them it was necessary to use the mean values for the dry-matter content of mammary tissue determined in Exp. 5. The results for Exp. 3 are in agreement with those for Exp. 5, since there was no difference in total dry weight between the glands from the two groups of rats while the water content differed in the same sense and by the same amount (10.6 %). In Exp. 4, however, in which the rats received a diet containing 0.67 % Na as against the 0.33 % Na content of the stock diet, the total dry weight of the glands from doca-treated adrenalectomized rats was somewhat higher than that of the glands from the sham-operated controls, the difference being just statistically significant. The water content of the glands from the former group was, however, again increased over the control values and to the extent of 10 %. In view of the fact that these results were obtained by rather indirect calculation it is doubtful how much importance should be attached to the apparent increase in mammary tissue in the doca-treated, adrenalectomized rats in Exp. 4, particularly as the probability that the difference was not due to chance is not very high. If the difference is indeed real, it may be connected with the effects of the increased sodium intake of these animals acting in conjunction with doca.

#### DISCUSSION

The experiments reported in this paper show that the water content of the mammary tissue of rats adrenalectomized 4 days after parturition and thereafter given daily injections of doca is significantly greater than that of the glands of normally lactating rats.

It may also be concluded from the values for total dry matter of the abdominal glands that the glands of the adrenalectomized rats undergo no further growth under the influence of daily injections of doca, the treatment serving to do no more, structurally speaking, than to maintain them in a condition analogous to that of the controls. In other words, the mammary enlargement observed in the operated, treated rats involved no hyperplasia, and may be ascribed entirely to increased hydration of the tissue.

It is not equally clear that the decrement in total 'milk-free' weight exhibited by the mammae of untreated adrenalectomized lactating rats was wholly due to the

converse process of dehydration. Histologically, most of the glands of the untreated adrenalectomized rats in Exp. 1 showed advanced involution, so that the weight decrement may have been partly due to dehydration of the tissues and partly to involution consequent upon adrenalectomy. It must also be remembered that we were unable to determine the lactose content of the milk present in the mammae of the untreated adrenalectomized rats, and in calculating the milk content of these tissues were forced to use the value for normal rats' milk on the assumption that the lactose content is unaffected by adrenalectomy. That such an assumption may be unjustified appears from the work of Levenstein [1937] who, on the basis of histological studies, deduced that the milk of untreated adrenalectomized rats contained less than the normal percentage of water. This conclusion is in keeping with the suggestion from our own results that the water content of the milk of adrenalectomized rats receiving high doses of doca is increased above normal. It seems possible, therefore, that the milk of adrenalectomized rats may contain more than the normal amount of lactose, and it would follow that our estimates of the net weights of the abdominal mammae of untreated adrenalectomized rats (Table 1) are low.

Our results hardly fit in with previous work on the relation between the adrenal cortex and the water content of tissues. A number of workers [e.g. Silvette & Britton, 1933; Silvette, 1934; Ponder & Gaunt, 1934-5] claimed that adrenalectomy increased the water content of certain tissues such as muscle and liver, though the effects in general were small and probably would not have survived statistical analysis. Later workers [Hegnauer & Robinson, 1936; Harrop, 1936; Muntwyler, Mellors, Mautz & Mangun, 1940] were able to show that the water content of the intracellular compartment of the body as a whole, or of certain tissues, increased after adrenalectomy.

On the other hand, Ziegler, Anderson & McQuarrie [1944] could observe no effect of 1 mg. of doca daily on the water content of the brain or heart muscle in the rat, while daily doses as high as 10 mg. per day per rat were found by Selye & Hall [1943] to cause no marked retention of water or tissue oedema. Other workers [see Loeb, 1941], on the contrary, report water retention by the body as a whole accompanied by an increase in blood and interstitial fluid volume under the influence of doca.

In any event the present work, so far as we are aware, provides the first demonstration\* that doca can increase the hydration of a specified tissue, though we do not suggest that, in these experiments, the hydration effect was limited to the mammary gland. In most of our experiments on replacement therapy with 3.0 mg. of doca daily in adrenalectomized lactating rats [Cowie & Folley, 1947*a*, *c*] the operated, treated rats have exhibited much larger increases in body weight during the experiment than sham-operated controls. It seems likely that much of this extra body-weight increment was due to water retention. The present work shows that part at any rate of the increased body water was located in the mammary gland.

The mammary gland may be considered as consisting of three compartments: intracellular, extracellular or interstitial, and alveolar compartments, the latter being a special extracellular, strictly speaking an 'extra-tissue', compartment. In the present work an attempt has been made to eliminate the effect of changes in the water

\* Since correcting the proofs of this paper we have seen a publication by Berman, Sylvester, Hay & Selye (1947) in which they report that treatment with deoxycorticosterone acetate increased the water content of the liver remnants in partially hepatectomized rats.

content of the alveolar compartment by determination of the milk content of the tissue. It has not been possible to differentiate between changes in hydration of the intracellular and extracellular compartments, so that we cannot be sure whether or not the increased tissue hydration observed in the mammae of doca-treated adrenalectomized rats is due to increased cellular hydration or to a condition of oedema.

Determinations of the distribution of water between the intracellular and extracellular compartments of various tissues (for discussion see Peters [1935]) reveal quite wide differences between different tissues, though in most cases the cells contain appreciably more water than the interstitial spaces. It seems reasonable to suppose, in the absence of definite data, that the same would hold for the mammary gland, and it is therefore unlikely that an increase in total water content amounting to over 10% of the original value could be due solely to an increase in what is probably the minority fraction, the interstitial water. The probability is that a good proportion of the observed increase is due to increased hydration of the epithelial cells.

It might be thought that the increased hydration of the mammary gland tissue would have an adverse effect on the functioning of the mammary gland—that this might be the reason why replacement therapy with doca, under our conditions, does not completely restore lactation. This does not appear to be the case, however, since Folley [1942] found that administration to intact lactating rats of as much as 10 mg. of doca daily had no deleterious effect on lactation as indicated by the growth of the young.

#### SUMMARY

1. The six abdominal mammary glands in rats adrenalectomized on the 4th day of lactation, and thereafter given 3.0 mg. of deoxycorticosterone acetate (doca) daily, were significantly heavier (on a 'milk-free' basis) at autopsy on the 17th day than those of sham-operated controls. Those of untreated adrenalectomized rats were much lighter in weight than the control glands.

2. Determinations of the total dry matter in the abdominal mammae, after correction for the dry matter of the milk retained in the alveoli, show that no cellular hyperplasia had occurred in the glands of the doca-treated adrenalectomized animals.

3. The water content of the mammary tissue (corrected for the water present in the alveoli) of the doca-treated animals was approximately 10.6% (approximately 7.4 units of percentage content) greater than that of control glands. It is believed that part at least of the increased water content was due to cellular hydration.

4. The findings are discussed in relation to the effect of doca on lactation.

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# A RAPID METHOD FOR VOLUMETRIC AND NUMERICAL ESTIMATION OF PANCREATIC ISLETS IN THE MOUSE

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Recent research on the islets of Langerhans makes evident the need for some way in which this tissue, and its insulin-producing capacity in the experimental animal, may be estimated quantitatively in a simple and yet reliable manner. Assay of the insulin content of the 'resting' pancreas, as an index of the mass of functional islet tissue, cannot be considered reliable, since the amount of insulin extracted will depend on the balance between production, liberation, and storage of the hormone.

Bensley [1911], using intravital staining (neutral red and janus green), showed that the total number of islets in the guinea-pig could be counted, but the technique was not used for quantitative volume estimation. Similarly, Clark [1913] developed a method for counting the islets of the human pancreas after perfusion of the organ with vital dye. Other methods have been employed for measuring the amount of islet tissue, and in the main they involve fixation and embedding of the pancreas, with volume estimation of a certain proportion of the sections cut in series. Opie [1900], Sauerbeck [1902] and Cecil [1912] carried out islet counts from sections of portions of the human pancreas; Heiberg [1906] estimated the total islet content, and later Ogilvie [1935, 1937] worked out the method in detail for the purpose of an extensive investigation; Susman [1942] employed a similar technique. Richardson & Young [1937] calculated the islet/acinar ratio in rats by examination of a high proportion of serial sections, and Cameron [1926] and Stosch [1940] estimated the islet content of the pancreas of the guinea-pig and the mouse respectively from stained serial sections.

More recently Haist & Pugh [1947] have stained the pancreas of small animals with intravital neutral red, pressed the tissue between plates, and projected the preparation on a screen. It is possible by this method, of course, to count the number of islets as shown by Bensley, but regarding volume estimation, the authors give no details concerning the thickness of the pressed tissue, and it must be assumed that the islets extend from plate to plate. The volume of islet tissue was calculated from the original volume of the pancreas, by comparing the areas of acinar and islet tissues on the screen.

If intravital staining is carried out according to Bensley, with perfusion by a dilute solution of dye, the pancreas is swollen to varying degrees in each animal, and thus the method, for volumetric purposes, involves the assumption that the acinar and islet tissues are swollen by proportionately equivalent amounts. The validity of this assumption may be queried. Moreover, intravital staining with neutral red does not give unequivocal results as regards differentiation between acinar and islet tissue. The calculation of islet volume by this method involves the original volume of the pancreas.

Whether this latter be estimated as volume or as weight, it is difficult to make allowance for any fat or lymphoid tissue included, and in the tissues of the perfused animal there is always a quantity of transuded fluid which adds to the weight or volume.

The optical method presented here allows of the measurement of the total pancreatic islet tissue in the mouse. It avoids the necessity for any histological treatment of the tissue, it is rapid, a minimum of apparatus is required, and a permanent record of the islets and acinar tissue is made.

#### METHOD

Dissection of the entire pancreas of the mouse can be carried out more efficiently by first removing the stomach, intestines and spleen *in toto*. The pancreas is then separated from the rest of the viscera, care being taken to isolate the entire organ, to dissect off all fat and lymph glands, and to prevent any drying of the tissues. The pancreas is immersed in normal saline, through which carbon dioxide (exhaled air) is bubbled. It sinks in normal saline, but any strands of fatty tissue still attached tend to float, making it easy to remove them.

After washing in normal saline saturated with carbon dioxide for about half a minute, the pancreas is placed in a drop of this fluid on a glass plate coated with black cellulose paint. A clean glass plate is placed over the black plate and the tissue pressed out between them. The distance between the plates, and therefore the thickness of the pressed tissue, is regulated by interposing small strips of cover-slip,  $180\mu$  in thickness, around the periphery, and clamping the plates firmly together by spring clips at the edges.

The islets are visible, through the transparent plate, as discrete, sharply outlined, white spots, varying in size, and distributed irregularly in the dark blue-grey areas of acinar tissue. A photograph of the preparation is made, at a known magnification, using reflected light from a 12 V. 2 amp. projector lamp, arranged to give fairly uniform illumination over the field. The photographs on thin film, half-tone, quarter plates, are given slight under-exposure with over-development to increase the contrast between the bright islets and the dark acinar tissue.

The negative photograph, when projected on a screen at a total magnification of 30-40 diameters, shows the islets as black shapes on a lighter acinar background. A finely lined grid is put in the projector with the negative, so that the image is divided into squares. The islet outlines are traced on paper in such a way that all the islets are drawn side by side to form a continuous area, the grid ensuring that no islets are missed.

The number of islets in the whole pancreas may be counted directly on the screen, and the total islet area, as drawn on the paper, is estimated with the planimeter. The magnification is known, and thus the presenting area of islet tissue in the original preparation may be calculated. The total volume of islet tissue is obtained by taking  $47\mu$  for islet thickness, an average figure estimated, as described below, from measurements of serial-section reconstruction.

The total volume of the pancreas may be determined accurately by drawing an outline, for convenience at a magnification less than that employed for the islets, and estimating the outlined area planimetrically. This gives the presenting area of the total pancreas, which, multiplied by the thickness of the pressed tissue,  $180\mu$ , gives the pancreatic volume.

## DISCUSSION

From serial sections of areas of pancreas, photographed before fixation, the identity of islet tissue with the white spots in the living preparation has been checked conclusively. Moreover, it is clear from careful comparison between the living material and the stained, sectioned material that such structures as ducts or blood vessels do not appear on the photographic negative, and are thus not confused with islets.

Reconstruction of a number of individual islets, from serial sections of fragments of three pancreases fixed while pressed flat between the glass plates, showed that the islets were flattened, and it could not be assumed that each islet in the pressed preparation existed as a cylinder extending through the  $180\mu$  thickness of the preparation. Measuring the area of each islet in each section planimetrically, the total volume of islet tissue in the series, sectioned at known thickness, was calculated. The volume of islet tissue as computed from a photograph of the fresh preparation was then found to be in fair agreement with that measured from the sections (1% variation), an average thickness of  $47\mu$  having been taken for the islets in the preparation.

While it is unnecessary to discuss the probable reasons for the difference in optical properties between living acinar and islet tissue under the conditions outlined above, it should be noted that the effect does not depend upon a particular wave-length of light. It has been found that a black surface, as the absorption surface, is superior to a surface of any other colour. If there is any air space between the tissue and the black surface, the result is poor, which may be due to total internal reflexion at the glass-air interface, with the emission of more light from the acinar areas than is the case when the absorption surface is immediately beneath the tissue. Best results are obtained when the black-painted surface is in contact with the pancreas, and not when the reverse side of the painted plate lies next to the tissue.

The angle of incidence of light on the surface of the preparation is of no particular importance, except that if the angle becomes too small direct reflexion increases, with the production of glare and the fogging of the photographic plate.

If a series of pancreases are prepared in this way, the translucency of the acinar tissue of the specimens washed only in normal saline is variable. Occasionally there is insufficient contrast with the islets, and this appears to be unrelated to the age or sex of the mouse, or to the temperature at which the pancreas is prepared. The opacity of acinar tissue seems to be related to the pH of the medium into which the organ is introduced, since the contrast between islet and acinar tissue is uniform and maximal when the saline is saturated with carbon dioxide.

Regarding the accuracy of the method, the objection may be raised that the islet volumes determined from the fixed and stained sections cannot be applied to the fresh tissue preparation. The pancreas does shrink as the result of fixation and embedding, but the greater part of the shrinkage occurs in the acinar tissue. This criticism may legitimately be brought to bear upon previous work, where islet and acinar volumes have been estimated from stained sections, and islet/acinar ratios determined from these results. Comparative measurements of acinar and islet tissue areas, first in the fresh preparation and later when the same material is sectioned and stained, show that the islets change in volume by only a small amount.

It is possible to estimate the thickness of the islets in groups, varying according to presenting surface area in the fresh preparation, but calculation on this basis shows that the variation from the total volume estimated, using an average islet thickness of  $47\mu$  (in total tissue thickness of  $180\mu$ ), is small as compared with the other errors inherent in the method. If the apparatus is standardized, the distance between the plates being fixed, the method gives a very good index of total islet volume when the presenting surface area only of the islet tissue is measured.

By determining the average islet thickness for a particular interplate distance, the method would appear to give a close approximation to the actual total islet volume.

This method, in its present form, is not applicable to animals other than the mouse. The rat pancreas, prepared in this way, shows some differentiation between acinar and islet tissue, but not sufficient for an accurate estimation.

#### SUMMARY

A method is presented whereby the total pancreatic islet tissue and pancreatic volume in the mouse may be estimated rapidly and accurately from a photograph of a fresh tissue preparation. Histological fixation and staining are not involved. The validity of the method, and comparison with other methods, are discussed.

This work is part of an investigation into the growth of pancreatic islets, carried out with the aid of a grant from the British Empire Cancer Campaign to Prof. F. G. Young. For the assistance thus provided I wish to express my thanks. In addition, I should like to thank Mr K. C. Richardson for his generous help and useful criticism, and Mr F. J. Pittock for his advice on photographic methods.

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# THE QUANTITATIVE DETERMINATION OF SMALL AMOUNTS OF PREGNANEDIOL IN HUMAN URINE

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Various methods have been described for the quantitative determination of pregnanediol in human urine. Of these the original procedure of Venning [1937, 1938], in which sodium pregnanediol glucuronide is extracted from the urine and weighed after purification, is still perhaps the most widely employed. This method, although somewhat time-consuming and laborious, gives satisfactory results when applied to urines containing more than c. 10–15 mg. of pregnanediol per 24 hr., but suffers from certain serious disadvantages where urines containing smaller amounts of the steroid are concerned. Thus, as pointed out by Astwood & Jones [1941], 'when only small amounts of material are present in the urine, the identity of the final product is sometimes questionable'. Furthermore, it may be necessary to use a full 24 hr. or even a 48 hr. specimen of urine for a single determination in order to obtain sufficient of the glucuronide to weigh accurately.

Other methods of determining pregnanediol as its glucuronide have been described by Allen & Viergiver [1941], Jayle, Crépy & Wolf [1943], and Bisset, Brooksbank & Haslewood [1947]. These methods would seem to be somewhat less laborious and considerably more sensitive than the Venning method, but they are open to criticism on the grounds of lack of specificity. A further disadvantage of all methods in which pregnanediol is determined as its glucuronide arises from the fact that, unless very special precautions are taken, hydrolysis of the latter by bacterial action may occur during the collection of the urine and subsequently.

A method of a different kind was developed by Astwood & Jones in 1941. In this method the urine is boiled with acid to hydrolyse the glucuronide and the free pregnanediol thus liberated extracted with toluene, purified and weighed. The originality of this method lies in the procedure used for the purification of the pregnanediol in the crude toluene extract. It was shown that from the toluene-soluble fraction, after removal of acidic substances by treatment with sodium hydroxide, nearly pure pregnanediol in almost quantitative yield can be obtained by precipitation from ethanolic solution with four volumes of water or dilute sodium hydroxide solution. The sensitivity of this method was considerably increased subsequently by Talbot, Berman, MacLachlan & Wolfe [1941], who estimated the purified pregnanediol colorimetrically by means of the yellow colour which it yields with concentrated sulphuric acid. Both groups of workers reported reasonably satisfactory recoveries of about 70% in short series of experiments in which sodium pregnanediol glucuronide was added to men's urine in varying amounts,\* but the evidence that either procedure

\* It seems possible that the recoveries obtained by these authors may actually have been somewhat higher than they themselves reported, since Marrian & Gough [1946] have shown that sodium pregnanediol glucuronide prepared and purified in the usual way [Venning & Browne, 1936] contains only about 80% of that compound.

would be dependable when applied to urines containing less than *c.* 10 mg. of pregnanediol per 24 hr. was not entirely satisfactory.

A somewhat simplified version of the Astwood & Jones method as modified by Talbot *et al.* has recently been developed by Guterman [1944, 1945] for the detection of pregnanediol in urine as a rapid means of diagnosing pregnancy.

What will for convenience be called the 'Astwood-Talbot' method has several advantages over other methods of determining urinary pregnanediol. The colour reaction which pregnanediol gives with concentrated sulphuric acid is extremely sensitive, thus permitting the accurate determination of small amounts of the substance; and although it is not a specific reaction for pregnanediol it was shown by Astwood & Jones and by Talbot *et al.* that many of the other urinary substances which give it are largely eliminated in the ethanol-water precipitation process of purification. Since the pregnanediol is determined in the free state, elaborate precautions to avoid bacterial hydrolysis of the glucuronide in the urine are unnecessary.

The objective of the work described in this paper was to develop an accurate, specific, convenient and rapid method for the determination of the pregnanediol (1–10 mg./24 hr.) in the urine of women during the luteal phase of the menstrual cycle. Furthermore, in order to permit duplicate determinations of pregnanediol and of other urinary constituents to be carried out it was necessary that the method developed should require not more than one-fifth or one-quarter of a 24 hr. specimen of urine.

This objective has been nearly, but not quite, attained. A procedure based on the Astwood-Talbot method has been elaborated which permits of the reasonably accurate determination of more than *c.* 0.4 mg. of pregnanediol in one-fifth of a 24 hr. urine specimen. Six determinations can be completed in two 8 hr. working days, and the method is such that it could be satisfactorily employed by a trained and competent laboratory technician.

#### APPARATUS AND MATERIALS

Quickfit & Quartz glassware, with interchangeable standard glass joints, was used throughout in order to avoid contamination of the urinary extracts with coloured or chromogenic material that might be dissolved out of rubber or cork stoppers.

The toluene used for the extractions was 'sulphur-free' and was distilled before use. Ethanol was purified by refluxing over sodium hydroxide and distilling twice.

Pregnane-3( $\alpha$ ), 20 $\alpha$ -diol was prepared from human pregnancy urine and purified *via* its diacetate. The sample used in the recovery experiments melted at 236–237° (corr.).

Sodium pregnane-3( $\alpha$ ), 20 $\alpha$ -diol glucuronidate was prepared from human pregnancy urine by the method of Venning & Browne [1936] and freed from ketonic glucuronides by the method of Sutherland & Marrian [1946, 1947]. The preparation used in the recovery experiments melted at 282–283° (corr.) with decomposition and evolution of gas. Samples of the glucuronidate were weighed out after exposure to moist air. As shown by Sutherland & Marrian [1947] material so treated is the *trihydrate* having the composition  $C_{27}H_{43}O_8Na \cdot 3H_2O$ .

For certain of the recovery experiments the toluene-soluble neutral fraction of acid-hydrolysed men's urine was required. This was prepared in the following way: 24 hr.

urine specimens from a number of normal men were pooled and heated to boiling, together with some toluene, acidified with one-tenth of its volume of concentrated hydrochloric acid, and the boiling continued for 10 min. After cooling the mixture was extracted three times with one-fifth volumes of toluene, emulsions being broken by filtration through a Buchner funnel with gentle suction. The combined toluene extracts were washed twice with one-sixth volumes of N sodium hydroxide, three times with one-sixth volumes of water, evaporated to a small volume on a hot plate, and finally taken to dryness under reduced pressure in the water-bath. The brown gummy material so obtained will be referred to as 'male urine neutral fraction'.

#### MODIFICATIONS MADE IN THE PROCEDURES OF ASTWOOD AND TALBOT

In consequence of a very large number of preliminary experiments, the details of all of which are not reported here, a number of modifications have been made in the original procedures as described by Astwood & Jones [1941] and by Talbot *et al.* [1941]. These modifications were designed to minimize the number of transferences from vessel to vessel during the procedure and to facilitate the rigid standardization of technique which experience has shown is essential if accurate and reproducible results are to be obtained with urines containing less than c. 10 mg. of pregnanediol per 24 hr. urine volume.

##### *Removal of acidic substances*

Astwood & Jones and Talbot *et al.* removed acidic and phenolic material from the toluene extract of the hydrolysed urine by boiling with methanolic sodium hydroxide and then filtering off the resulting precipitate of sodium salts. In the experience of the present authors it is equally effective and much simpler to remove acidic and phenolic material by washing the toluene extract in a separating funnel with aqueous sodium hydroxide.

##### *Purification of pregnanediol from the neutral fraction by precipitation from ethanol*

The accuracy and specificity of the Astwood-Talbot method are very largely dependent upon the efficiency of the precipitation process in separating pure pregnanediol quantitatively from the neutral toluene-soluble fraction of the hydrolysed urine. Accordingly, considerable attention has been paid in the present work to investigating the optimal conditions for carrying out the precipitation and for collecting the precipitated pregnanediol.

*Collection of the precipitated pregnanediol.* In the Astwood & Talbot procedures and also in the method of Guterman [1944] the precipitated pregnanediol was collected by filtration. To facilitate the quantitative collection of the small amounts of precipitate and in order to avoid as far as possible transference of the material from vessel to vessel, the precipitation process in the present work has been carried out in centrifuge tubes and the precipitate collected by centrifugation. At first some difficulty was experienced in getting the relatively light pregnanediol crystals to pack sufficiently tightly in the tubes to permit the siphoning-off of the supernatant solution. This difficulty was, however, overcome by adding to the precipitation mixture before centrifugation a small amount of the filter-aid 'Hyflo-Super Cel', which effectively entrained the precipitated pregnanediol.



*Number of precipitations and volume of precipitation mixture.* Astwood & Jones [1941] purified the pregnanediol from the neutral fraction by one precipitation with four volumes of N/10 sodium hydroxide followed by one with four volumes of water. Talbot *et al.* [1941] and Guterman [1944] used a single precipitation with four volumes of N/10 sodium hydroxide. In the experience of the present authors, however, one precipitation with sodium hydroxide and two with water are required in order to obtain the pregnanediol in a satisfactory state of purity. This multiple precipitation technique lengthens the process somewhat, but the loss of time is more than compensated for by the resulting increase in specificity which results.

Numerous preliminary experiments showed that the most satisfactory results were obtained when the neutral fraction from one-fifth of a 24 hr. specimen of urine was dissolved in 4 ml. of ethanol and precipitated with 16 ml. of N/10 sodium hydroxide or water.

*Rate of cooling after precipitation in hot solution.* The efficiency of the precipitation process has been studied in many series of recovery experiments in which varying amounts of pure pregnane-3( $\alpha$ ), 20 $\alpha$ -diol were added to quantities of 'male urine neutral fraction' each corresponding to one-fifth of a 24 hr. urine specimen. In the early experiments of this kind it was found that satisfactory recoveries (80% or better) were obtained when more than about 4 mg. of pregnanediol were present (corresponding to 20 mg./24 hr.), but that with smaller amounts of pregnanediol the recoveries were very much lower and very irregular.

The great irregularity of the recoveries suggested that some important variable factor in the purification process was not being properly controlled. Since the rate of cooling of the mixture after precipitation in hot solution might be expected to determine the size of the pregnanediol crystals it seemed possible that this might be the uncontrolled factor.

This possibility was investigated in two series of recovery experiments in which varying amounts of pure pregnane-3( $\alpha$ ), 20 $\alpha$ -diol were added to portions of 'male urine neutral fraction', each of which was equivalent to one-fifth of a single 24 hr. specimen. The pregnanediol in these mixtures was purified by the triple precipitation procedure, which was as described below (p. 252) with the exception that the cooling conditions following each precipitation were varied. In one series of experiments the precipitation mixtures were cooled rapidly by immediate immersion in an ice-bath and were then left in the refrigerator overnight before centrifugation. In a second series, which were duplicates of the first, the mixtures were cooled slowly by transferring them in beakers of water at 75° to an incubator at 37° where they were allowed to remain for 2 hr. They were then cooled in the refrigerator for 30 min. and centrifuged.

The results\*, which are shown in Table 1, are quite conclusive. It will be seen that rapid cooling of the precipitation mixtures gave lower and much more irregular recoveries of pregnanediol than were obtained by the slow-cooling technique. It will also be seen that the difference in recovery due to the rate of cooling was most marked with amounts of pregnanediol corresponding to less than c. 20–25 mg./24 hr.

Later experiments, which are not reported here, have shown that the recoveries by the slow-cooling technique are unaffected by the omission of the short period of refrigeration before centrifugation. It has also been found that a longer period at 37°

\* A preliminary paper dealing with these findings was read to the Society for Endocrinology on 29 May, 1947.

than 2 hr. does not lower the recoveries. In the method finally adopted (p. 252) the actual periods during which the precipitation mixtures are incubated have been adjusted to fit a working day of convenient length.

Table 1. *The effect of varying the rate of cooling after precipitation on the recovery of pregnanediol added to 'male urine neutral fraction'*

'Male urine neutral fraction'	Pregnanediol added (mg.)	Pregnanediol recovered (mg.)		Pregnanediol recovered corrected for 'male urine blank' (%)	
		Rapid cooling	Slow cooling	Rapid cooling	Slow cooling
A	0.00	0.056	0.056	—	—
	0.00	0.012	0.055		
	0.00	0.026	0.040		
		0.031		0.050	
B	0.00	0.017	0.040	—	—
	0.00	0.015	0.042		
	0.00	0.010	0.037		
		0.014		0.040	
A	0.27	0.032	0.123	0	17
	0.27	0.012	0.092		
	0.27	0.025	0.072		
		0.023		0.096	
B	0.40	0.016	0.33	1	75
	0.40	0.021	0.38		
	0.40	0.015	0.31		
		0.017		0.34	
A	0.53	0.43	0.45	66	84
	0.53	0.42	0.46		
	0.53	0.37	0.57		
		0.41		0.49	
B	0.80	0.63	0.86	89	99
	0.80	0.82	0.82		
	0.80	0.80	0.83		
		0.75		0.84	
A	4.0	4.0	4.2	86	101
	4.0	3.9	4.1		
	4.0	2.5	4.0		
		3.5		4.1	

In view of these findings it is of some interest to consider the precipitation and cooling techniques of previous workers who have used the Astwood-Talbot method. Astwood & Jones [1941] do not appear to have controlled the temperature at which precipitation was carried out very exactly, but they did allow the precipitation mixtures to cool to room temperature before cooling in the refrigerator. Such a procedure in an American laboratory, where 'room temperature' may be 25° or more might be said to provide 'slow cooling'; in the average British laboratory in winter time, however, the procedure might very well give quite rapid cooling. Neither Talbot *et al.* [1941] nor Guterman [1944] controlled the temperature of precipitation, and since in both cases the precipitation mixtures were transferred directly to the refrigerator or into an ice-bath, their procedures must have undoubtedly involved rapid cooling. It can be concluded that the procedures used by all these workers would be liable to give low and erratic results with urines containing less than c. 20 mg. of pregnanediol per 24 hr.

#### *Sulphuric acid colour reaction*

The finally purified pregnanediol obtained by the precipitation process from the urine of pregnant and of non-pregnant women has occasionally been found to be contaminated with traces of a blue pigment. The nature of this pigment has not been

investigated, but it is suspected that it may be of dietary origin. The presence of this pigment seriously interferes with the sulphuric acid colour reaction and it is therefore necessary to remove it before carrying out the reaction. This can be effectively done without loss of pregnanediol by warming in ethanolic solution with charcoal. In order to maintain a rigid uniformity in procedure this treatment with charcoal has been adopted as a routine whether the pigment is present or not.

Talbot *et al.* [1941] carried out the colour reaction by allowing the purified pregnanediol to stand with 10 ml. of concentrated sulphuric acid at room temperature for 20 min. In the present work, in order to standardize conditions as far as possible, the colour development has been carried out in a water-bath at 25° instead of at 'room temperature'.

#### METHOD FINALLY ADOPTED FOR THE DETERMINATION OF PREGNANEDIOL IN URINE

A 24 hr. specimen of urine collected with 5 ml. of toluene as preservative is made up to 2500 ml. and duplicate 500 ml. samples removed. Each sample is treated as follows: It is placed in a 1000 ml. flask and after the addition of 100 ml. of toluene brought to boiling point under a reflux condenser. To the boiling mixture is added down the condenser 50 ml. of concentrated hydrochloric acid (A.R.), and the boiling continued for exactly 10 min. The flask is then rapidly cooled in cold water and the contents transferred to a separating funnel of 750 ml. capacity. After shaking and allowing the urine layer to separate, the latter is run off into the original flask and the layer of toluene and emulsion filtered with gentle suction through a Whatman No. 1 paper on a Buchner filter funnel. The urine layer is then returned to the separating funnel and extracted twice more with 100 ml. portions of toluene, each toluene and emulsion layer being filtered in succession through the same filter funnel. The combined filtrates are then transferred to a clean separating funnel, and after running off the small urine layer that separates, the toluene extract is washed twice with 100 ml. portions of N sodium hydroxide and twice with 100 ml. portions of water. The washed toluene extract is run into a 500 ml. round-bottomed flask and is evaporated nearly to dryness on an electric hot plate and then taken completely to dryness under reduced pressure on a boiling-water bath.

The dry residue is transferred quantitatively with warm ethanol to a 20 ml. conical centrifuge tube and the ethanolic solution evaporated to dryness in a water-bath under a stream of air. To the residue in the tube are added exactly 4.0 ml. of ethanol and the tube is placed in a beaker of water maintained at 75°. After stirring with a glass rod for 1 min. to obtain complete solution, 16.0 ml. of N/10 sodium hydroxide are added drop-wise from a burette during 3 min. with stirring, the last 1-ml. being used to wash down the stirring rod into the tube. After a further 1 min. at 75°, the beaker of water containing the tube is transferred to an incubator at 37° and left overnight. Approximately 8–10 mg. of 'Hyflo-Super Cel' (Johns-Manville Co. Ltd.) are added and the mixture stirred with a glass rod. The rod is washed down into the tube with 1 ml. of a 1 : 4 (v/v) ethanol-water mixture and the tube is then centrifuged for 1 hr. (1500 r.p.m.; radius of centrifuge head: 15 cm.). The supernatant solution is finally sucked from the precipitate with the aid of a fine glass tube attached to a slowly running water-pump.

The second and third precipitations are carried out as described above, except that water instead of sodium hydroxide solution is used, and the incubation periods are reduced for convenience to 2 hr. No additional filter-aid is added before the centrifugations following the second and third precipitations.

To the final precipitate are added 5 ml. of ethanol and the pregnanediol dissolved by warming with stirring at about 75°. 'Norite' charcoal (c. 1–2 mg.) is then added and the warming continued for 2 min. The mixture is filtered through a small filter (Whatman No. 1 paper) into a test-tube of 1 in. diameter, the centrifuge tube and filter being washed three times with 2 ml. portions of warm ethanol. The filtrate and washings in the tube are evaporated in a water-bath under a stream of air and the residue finally dried by leaving the tube in a vacuum desiccator over calcium chloride for several hours.

The colour reaction is carried out with not more than c. 0.5 mg. of the finally purified product. If, therefore, the amount of the latter appears on inspection to be in excess of 0.5 mg. a suitable aliquot portion is removed after solution in a known volume of ethanol. To the dry pregnanediol 10.0 ml. of concentrated sulphuric acid (A.R.) are added from a burette, and the tube is left in a water-bath at 25° for 20 min. with occasional shaking. The intensity of the yellow colour produced is measured in a 'Spekker' photoelectric absorptiometer using a 'spectrum violet' No. 601 light filter.

The absorptiometer readings are interpreted by reference to a calibration curve made with known amounts of pure pregnane-3( $\alpha$ ), 20 $\alpha$ -diol varying from 0.1 to 0.5 mg. It is advisable to construct a fresh calibration curve for each batch of unknowns.

#### RECOVERY EXPERIMENTS

The accuracy of the finally adopted method was tested in a long series of recovery experiments in which pure sodium pregnanediol glucuronide was added in varying amounts to men's urine. The validity of such tests of accuracy depends upon two assumptions: (a) that all the pregnanediol in human urine is present as the glucuronide, and (b) that women's urine contains no substances which are not present in men's urine which would interfere with the determination of pregnanediol. Further work will be necessary to see whether these assumptions are justifiable or not.

Twelve 24 hr. specimens of urine were collected from four normal men. Each specimen was made up to 2500 ml. and four 500 ml. samples removed. To each of two of these samples was added an identical amount of sodium pregnanediol glucuronide dissolved in 80% ethanol, the other two samples being retained for working-up as 'male urine blanks'. All four samples from each specimen were then treated as described in the preceding section (pp. 252, 253). The results in Table 2 show that a pregnanediol content of c. 2 mg./24 hr. is a critical one, above which recoveries are excellent, but below which they are poor.

That the loss of pregnanediol when less than c. 2 mg./24 hr. is present in the urine occurs mainly during the precipitation process rather than during the hydrolysis or extraction seems to be clear from the results shown in Table 1. This loss must be largely due to an effect of other substances in the neutral fraction upon the solubility of pregnanediol in 20% ethanol, since experiments in pure solution have shown that

80–95 % of pregnanediol can be removed after the triple precipitation process when it is present in amounts corresponding to as little as 0.5 mg./24 hr. It is possible, therefore, that the critical concentration of pregnanediol below which recoveries are unsatisfactory may vary somewhat with different urines. However, the results reported here with a number of different specimens of men's urine suggest that this critical concentration is probably not far from 2 mg./24 hr. in the majority of cases.

Table 2. *Recovery of pregnanediol after the addition of sodium pregnanediol glucuronide to men's urine*

Men's urine specimen	'Male urine blank' as apparent pregnanediol in $\frac{1}{2}$ of 24 hr. specimen (mg.) (av. of duplicates)	Pregnanediol added as glucuronide to $\frac{1}{2}$ of 24 hr. urine specimen (mg.)	Pregnanediol recovered (mg.)		Pregnanediol recovery (corrected) (%)
			Apparent	Corrected for blank	
C4	0.016	0.2	0.017	0.001	0
		0.2	0.012	—	0
A3	0.008	0.2	0.021	0.013	7
		0.2	0.047	0.039	20
B2	0.024	0.2	0.060	0.036	18
		0.2	0.045	0.021	11
D4	0.035	0.4	0.32	0.29	72
		0.4	0.33	0.29	74
B3	0.015	0.4	0.28	0.27	67
		0.4	0.29	0.28	69
A2	0.018	0.4	0.35	0.33	82
		0.4	0.35	0.33	82
A4	0.044	1.0	0.99	0.95	95
		1.0	0.98	0.93	93
C3	0.019	1.0	0.94	0.92	92
		1.0	0.96	0.94	94
D2	0.077	1.0	1.0	0.92	92
		1.0	0.98	0.90	90
B4	0.030	2.0	1.9	1.9	95
		2.0	2.0	2.0	100
D3	0.017	2.0	2.0	2.0	100
		2.0	1.9	1.9	95
C2	0.026	2.0	1.9	1.9	95
		2.0	1.9	1.9	95

#### SPECIFICITY OF THE METHOD

The sulphuric acid colour reaction is not specific for pregnanediol; many other steroids give similar colours with varying intensities. The specificity of the method as a whole therefore depends upon the completeness with which other chromogenic steroids originally present in the urine are eliminated in the extraction and purification process.

Some data relevant to this extremely important point have been presented by previous workers. Thus Astwood & Jones [1941] showed that cholesterol and androsterone were completely eliminated by their double precipitation process when present in amounts not exceeding 16 and 8 mg. per litre respectively, while Talbot *et al.* [1941] showed that dehydroisoandrosterone in amounts up to *c.* 10 mg. per litre

caused no interference. In the course of present work additional relevant data have been accumulated, but since these data are still incomplete they will be referred to at the present time only briefly.

Androsterone, *iso*androsterone, and pregnan-3( $\alpha$ )-ol-20-one are not completely eliminated by the triple precipitation process used, but providentially they are so much less powerfully chromogenic than pregnanediol in the sulphuric acid reaction that their presence in the final product, except in abnormally large amounts, does not introduce any serious error into the pregnanediol determination. Dehydro*iso*-androsterone and pregnane-3( $\alpha$ ), 17, 20-triol, on the other hand, are very powerful chromogens, but, providentially again, they seem to be very readily eliminated in the precipitation process. Any possible interference by the pregnanetriol is doubly safeguarded against by the fact that this compound would be largely decomposed during the initial hydrolysis of the urine with acid.

These preliminary findings provide hope that the method may be reasonably specific for pregnanediol when applied to normal urines, but before definitely concluding that it is indeed so it will be necessary to carry out further experiments with other steroids likely to be present. At the present time the method cannot be recommended for pathological urines containing abnormally high concentrations of neutral 17-ketosteroids or of other neutral steroids of adrenal origin. In such cases the pregnanediol present might be considerably overestimated. In passing, it may be remarked that any of the methods in which pregnanediol is determined as its glucuronide are also likely to give fictitiously high results when applied to urines containing abnormally high concentrations of steroids of adrenal origin, since certain of the latter are probably excreted as glucuronides also.

#### DISCUSSION

The methods of urinary pregnanediol determination which have hitherto been described are either insufficiently sensitive or insufficiently specific to permit of strictly quantitative studies being carried out upon pregnanediol excretion during the menstrual cycle and during the early stages of pregnancy. The method described here should make such studies more nearly possible, and in particular should be of some value in the investigation of causes of female sterility.

Although the method was not designed for use as a means of pregnancy diagnosis, it may be useful for this purpose, and because of its more quantitative nature it may prove to be less subject to both positive and negative errors than the more rapid Guterman [1944, 1945] method. In view of the widespread interest in the latter it would perhaps not be out of place to discuss in the light of the findings in the present work some of the possible sources of the negative and positive errors to which the method seems to be subject [cf. Reinhart & Barnes, 1946].

In his latest paper on the method Guterman [1945] has arbitrarily fixed on an intensity of colour in the sulphuric acid reaction corresponding to 6-8 mg. of pregnanediol per 24 hr. as the lower limit for a positive reaction. It seems probable that at such levels of pregnanediol excretion the unsatisfactory cooling conditions in the precipitation process in Guterman's procedure might lead to low and variable yields of pregnanediol and thus to false negative results. It is likely that a controlled 'slow

cooling' technique in the precipitation process might eliminate some at least of these false negatives.

The findings in the present work suggest that false positives might be caused by the presence of abnormally large amounts of the weakly chromogenic saturated neutral 17-ketosteroids which are incompletely removed even by a triple precipitation procedure, or by the incomplete elimination in the single precipitation of the Guterman method of the strongly chromogenic dehydroisoandrosterone. In this connexion it is noteworthy that Morrow & Benua [1946] recorded false positives in a case of arrhenoblastoma which was excreting 59 mg. of 17-ketosteroids per 24 hr. It is doubtful whether false positives caused by the presence of abnormally large amounts of 17-ketosteroids could be entirely eliminated by any simple modification in the precipitation process, but it is likely that their number could be reduced if a double instead of a single precipitation procedure were to be adopted.

In conclusion, one may perhaps question whether it is justifiable to accept as diagnostic of pregnancy any arbitrarily fixed low level of pregnanediol excretion associated with amenorrhea. It must be remembered that little is known at the present time about the metabolism of progesterone, and there are in fact few reasons to believe that pregnanediol is even the main metabolic end-product of the latter. The low yields of urinary pregnanediol obtained after the administration to human subjects of progesterone raise the possibility that the latter may be largely metabolized in the body by other routes. It is therefore questionable whether the level of pregnanediol excretion provides a reliable indication of the progesterone production in the body, as has been so widely assumed.

As pointed out by Reinhart & Barnes [1946], it is possible that the greatest source of error in the Guterman test is the 'individual variations in the metabolism of progesterone, both in the pregnant and the non-pregnant woman'.

#### SUMMARY

A procedure based on the methods of Astwood & Jones [1941] and Talbot *et al.* [1941] has been elaborated which permits of the reasonably accurate determination of more than c. 0.4 mg. of pregnanediol in a fifth of a 24 hr. sample of human urine.

This work was carried out at the request of the Committee on Human Fertility of the Medical Research Council. The authors are indebted to the Medical Research Council for a grant from which the expenses of the work were defrayed and for personal grants to two of them (N.G. and I.F.S.). They are also indebted to their colleagues who co-operated in the collection of urine and to Miss E. Sutherland who prepared the sample of pure sodium pregnanediol glucuronidate.

Part of this work was carried out by I.F.S. during the tenure of a Vans Dunlop Scholarship in the Department of Obstetrics and Gynaecology.

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# THE INFLUENCE OF TESTOSTERONE PROPIONATE ON PIGMENTATION OF THE MAMMARY NIPPLES IN FEMALE MICE

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In recent experiments on the influence of testosterone propionate on tumours induced by carcinogens, a striking pigmentation of the mammary nipples in female mice of several strains was observed. The nipples were easily visible against the brighter background of the abdominal coat, while they were hardly noticeable in the untreated mice of the same strains. In previous experiments [Flaks & Ber, 1938, 1939*a, b*], in which albino mice were treated with very large doses of testosterone propionate, these changes were not noticed, although the mice were maintained for a period of nearly 12 months.

It is known from earlier work that injections of oestrogens in guinea-pigs and humans induced hyperpigmentation of the nipples [Lipschutz & Merino, 1932; Watrin, 1934; Hamilton, 1939; Lisser, 1940; Davis & Boynton, 1941; Byron & Katzen, 1941]. Local application of oestrone and stilboestrol was also shown to cause hyperpigmentation of the nipples in male guinea-pigs [Fierz, 1939].

The influence of androgens on pigmentation was reported by Hamilton & Hubert [1938]: the skin in castrated men, which is generally pale, became darker after treatment with androgenic hormones. Edwards, Hamilton, Duntley & Hubert [1941] found that the melanin content of the skin in eunuchs is subnormal. Treatment with androgens resulted in an increase of the pigment. Forbes [1942] described hair changes and pigmentation of the mammary nipples in rats with implanted pellets of sex hormones. In these experiments a number of oestrogens and androgens were employed. All the oestrogens, but only androsterone among the androgens, caused a partial pigmentation of the fur of albino rats and alopecia in dark grey-brown rats. Testosterone propionate and dipropionate, and androsterone, did not induce pigmentation of the nipples. In addition, a few rats of the albino and dark grey-brown strains with diethylstilboestrolmethyl-ether pellets showed either black pigmentation of the capsule around the pellet or similar changes in the male mammary gland. Kirshbaum & Pfeiffer [1941] showed that local application of testosterone propionate induced deposition of melanin in the bill of the sparrow.

## MATERIAL AND METHODS

One hundred and eighty mice of four strains were employed in the experiments: CBA-black agouti, C3H-dark grey-brown, Strong A-albino and mixed stock (black, brown and albino). All mice were virgins, about 3 months old, except two breeding C3H mice which were approximately 12 and 17 months old. Seventy mice were injected, under the skin of the abdomen, with a 2.5% solution of testosterone propionate in arachis oil. Each mouse received weekly injections of 6.25 mg. of

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testosterone during 2-12 weeks. The total dose varied from 12.5 to 75.0 mg. Two mice were given about 100 mg. in 4 weeks. In twenty mice 20 mg. tablets were implanted under the skin of the abdomen or into the thigh. Each received from one to four tablets. The rate of absorption from one tablet amounted to about 4 mg. per month.

The experimental and control mice received 1 mg. of methylcholanthrene either by subcutaneous injection or in pellet form, except ten mice from Exps. 4 and 5. The treatment of experimental mice with testosterone was started on the same day. The skin of the abdomen was epilated with barium sulphide to show the reaction of the nipples.

#### EXPERIMENTS

*Exp. 1.* Five black female mice of mixed stock were treated with testosterone propionate during 12 weeks. Five mixed stock females of the same coat colour received an equal amount of pure arachis oil. After 12 weeks, the black coat of the treated mice became brownish black. The mammary nipples, which had been almost invisible in these virgin females, became about 2 mm. long and black. No changes were observed in the controls.

Five brown and five sandy females of the same stock, which were treated with testosterone in the same way, showed a marked lengthening of the nipples, but no abnormal pigmentation. No changes were seen in the control mice.

*Exp. 2.* Forty CBA mice, twenty females and twenty males, were treated with testosterone propionate, receiving 62.5 mg. in 10 weeks. Forty control mice, twenty females and twenty males, received injections of pure arachis oil. Three weeks after the start of the treatment (18.75 mg. injected) the female mice showed dark-stained, almost black, elongated nipples, which protruded above the abdominal fur. These changes persisted during the lifetime of the animals. No changes were seen in the treated males or in the control females.

*Exp. 3.* Ten mixed stock black or black-white female mice were implanted with a tablet of testosterone propionate into the thigh. Ten females of the same coat colour served as controls. Four weeks later all nipples in the treated mice showed a very marked elongation and were of a deep black colour. No changes were seen in the controls (Pl. 1, fig. 1).

Five mixed stock brown and five albino females were treated in the same way as the black mice. No colour changes of the nipples were observed. Elongation of the nipples, however, occurred in all treated females, irrespective of their coat colour.

*Exp. 4.* Eight CBA mice of one litter, six females and two males, were divided into two groups. Three females and one male were implanted with two tablets of testosterone propionate under the skin of the back. Three females and one male served as controls. Between the second and third week the treated females showed a considerable lengthening and hyperpigmentation of the nipples (Pl. 1, fig. 2). The controls did not show these changes.

*Exp. 5.* One C3H female, 17 months old, bearing an early mammary tumour was injected with 100 mg. of testosterone propionate during 4 weeks. Eight weeks after the start of the treatment the nipples were black and about 2 mm. long. Histological examination of a nipple showed accumulation of dark brown pigment in the epithelial layers. Accumulation of melanophores could be seen in the underlying connective tissue around the main duct (Pl. 2, figs. 3 and 4).



FIG. 1. Exp. 3. Mixed stock female mice. Control mouse on the left; treated mouse on the right, 50 days after implantation of 20 mg. of testosterone propionate. Masculinization and pigmentation of the mammary nipples.  $\times \frac{3}{4}$ .



FIG. 2. Exp. 4. CBA female mice. Control mouse on the left; treated mouse on the right, 28 days after implantation of 40 mg. of testosterone propionate. Masculinization and hyperpigmentation of the mammary nipples.  $\times \frac{1}{4}$ .

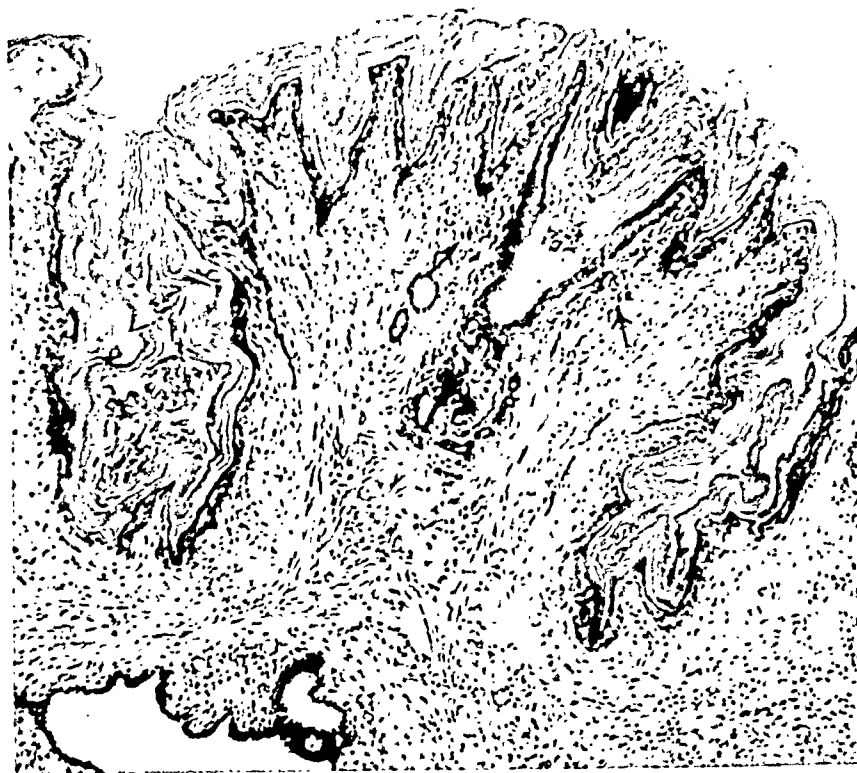


FIG. 3. Exp. 5. Mammary nipple of a C3H female mouse injected with 100 mg. of testosterone propionate, 60 days after the beginning of treatment. Arrows show accumulation of pigment. Fixed in Susa, H.E.  $\times 85$ .

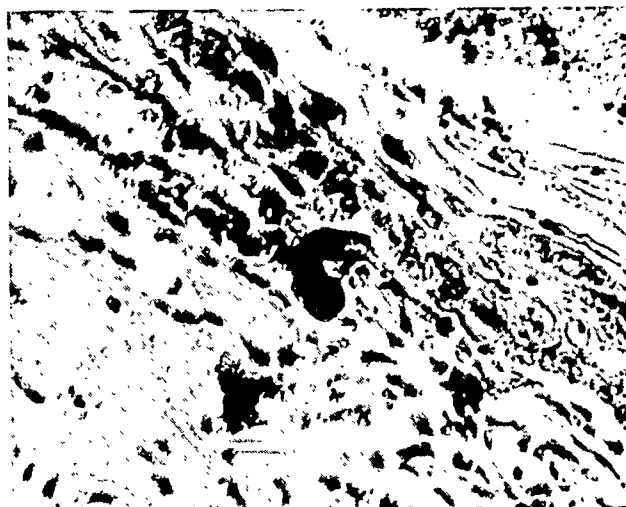


FIG. 4. Part of Fig. 3. Melanophores under the epitholium of the mammary duct and accumulation of free melanin granules in the epitholium and in the connective tissue.  $\times 450$ .

Another C3H female, 11 months old, with a mammary tumour, was implanted with two tablets of testosterone propionate. Four weeks later the nipples showed hyperpigmentation, although of a lesser degree than that of the previous mouse.

*Exp. 6.* Ten Strong-A albino females were implanted with two tablets of testosterone propionate under the skin of the abdomen. Ten females were employed as controls. After 14 weeks the treated mice showed only elongation of the nipples.

#### DISCUSSION

The earliest changes in pigmentation which could be detected with the naked eye were visible after 2 weeks of treatment. It is not known how much of the injected androgen was absorbed during that period. The amount absorbed in 2 weeks from one implanted tablet amounted to about 2 mg. A minimum amount of 4 mg. was required to induce pigmentation of the nipples.

Testosterone propionate, when injected subcutaneously under the nipple itself, exerted a more pronounced influence than when implanted far away from the nipple. This may be due to the greater concentration of testosterone in the region of injection.

There is uncertainty as to the mechanism of pigment formation *in vivo*. Figge & Allen [1941] investigated the influence of hormones on melanin formation, and found that the addition of oestrone to a tyrosin-tyrosinase mixture delays melanin formation *in vitro*. Figge [1939] postulated that 'melanin must be regarded as a natural reduction-oxidation system and that its very presence and colour may become a valuable intracellular indicator'. The marked pigmentation of the nipples in female mice treated with testosterone in the present experiments indicates that a chemical process of unknown nature induced formation of a considerable amount of melanin. The intensity of the pigmentation in the nipples seems to show that oxidation processes were involved. The deep black 'oxidized melanin' may be the indicator of the oxidation processes which took place. No pigmentation could be induced in albino mice. This shows that the necessary substrate for the pigment formation is present only in dark-coloured mice.

It is not known whether testosterone propionate has a direct or indirect influence on pigment formation *in vivo*. A direct influence cannot be excluded because of its direct influence *in vitro*, as shown by Hamilton [1940]. An indirect influence, however, may also take place because of the atrophy of the adrenals induced by high doses of testosterone. Deoxycorticosterone was shown to be an inhibitor of melanin formation [Hamilton, 1940]. The atrophy of the adrenals, which removes the influence of deoxycorticosterone, may enhance melanin formation. This may play an important part in the hyperpigmentation of the nipples in mice.

#### SUMMARY

Testosterone propionate induced a marked pigmentation of the mammary nipples in dark-coated female mice. The earliest changes were detected after 2-3 weeks' treatment. These changes were not observed in albino mice nor in males of the strains examined.

The author is indebted for the generous gift of Testoviron to Mr E. J. T. Goddard and Mr T. E. Riddle of British Schering Ltd., and to Dr W. A. R. Thomson of Boots Pure Drug Co., Ltd., for the testosterone propionate tablets.

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# THE INFLUENCE OF GONADAL HORMONES ON THE COMPOSITION OF THE BLOOD AND LIVER OF THE DOMESTIC FOWL

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It was suggested in a previous paper [Common, Rutledge & Bolton, 1947] that the increase in the fowl's serum riboflavin evoked by oestrogen cannot easily be explained as an outcome of mobilization and withdrawal of riboflavin from liver reserves. The relevant observations have been repeated and extended to include observations on the total dry matter, protein and fat contents of the livers of the experimental birds. The influence of thyroxine treatments on the effects of gonadal hormones was also investigated. The results of these two experiments form the subject of the present paper.

The general technique has been described in our previous paper. The experimental birds were sexually immature White Wyandotte pullets of the same strain as those in all the previous experiments, except Exp. 6 [Common, Rutledge & Hale, 1948]. All the pullets in any experiment were given the same amount of food daily. In all cases the gonadal hormones used were oestradiol dipropionate (Ovocyclin, Ciba) and testosterone propionate (Perandren, Ciba). The total amounts of oil injected into the different pullets were equalized by including suitable amounts of arachis oil.

The analytical methods have been described previously, except for the following additional methods employed in the present study. Dry matter was determined by drying to constant weight at 100° C. Crude protein was determined by macro-Kjeldahl. Fat was determined titrimetrically as tristearin by the modification of von Liebermann's method described by McCance & Shipp [1933]. Serum citric acid was determined by the method of Pucher, Sherman & Vickery [1936], using a Spekker absorptiometer in conjunction with a Wratten 47A filter.

## EXPERIMENTAL

### *Experiment 7*

In this experiment the hormonal treatments were again aimed to simulate in the sexually immature pullet the various changes taking place as the pullet passes from sexual immaturity to the laying of the first few eggs. Six equal doses of the hormones were injected intramuscularly on alternate days, the pullets being killed after an experimental period of 12 days.

The treatments and the basic experimental data are set out in Table 1. In so far as they relate to hypertrophy of the oviduct, serum calcium, plasma phosphorus fractions and serum riboflavin, the data in general confirm the previous observations.

The usual changes in haematocrit value and blood volume were also observed, but it may be remarked that when these data are used to calculate total volumes of



Table 1. *Effect of gonadal hormones on pullets*

	Pullet 53	Pullet 54	Pullet 55	Pullet 56	Pullet 57	Pullet 58	Pullet 59	Pullet 60
Dosage of oestradiol dipropionate (mg.)	Nil	6 x 1	6 x 2	6 x 4	Nil	6 x 1	6 x 2	6 x 4
Dosage of testosterone propionate (mg.)	Nil	Nil	Nil	Nil	6 x 0.75	6 x 0.75	6 x 0.75	6 x 0.75
Live weight, initial (kg.)	1.10	1.11	1.05	1.17	1.15	1.16	1.10	1.17
Live weight, final (kg.)	1.36	1.39	1.31	1.43	1.42	1.44	1.43	1.48
Ovary: State	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent
Weight (g.)	0.32	0.24	0.36	0.24	0.32	0.36	0.25	0.30
Oviduct: Weight (g.)	0.31	6.5	9.2	12.9	0.35	20.1	17.8	17.3
Crude blood volume (ml.)	46.2	50.4	49.8	74.5	42.5	55.5	53.3	63.2
Haematocrit value	29.9 (28.2)*	27.0 (26.0)	24.5 (23.8)	19.6 (30.6)	35.0 (26.2)	31.1 (26.5)	28.4 (26.4)	24.0 (26.2)
Serum Ca (mg./100 ml.)	11.9 (12.5)	22.7 (11.9)	49.8 (12.2)	102.6 (12.2)	11.5 (12.2)	32.7 (12.0)	64.8 (12.0)	98.4 (11.1)
Plasma acid-soluble P (mg./100 ml.)	7.8 (7.3)	7.8 (7.2)	17.6 (6.8)	20.1 (6.8)	8.3 (7.3)	10.9 (7.1)	22.7† (6.5)	23.0† (7.3)
Plasma inorganic P (mg./100 ml.)	7.0 (6.3)	8.6 (6.1)	16.0 (5.7)	18.1 (6.6)	7.0 (6.7)	9.5 (6.1)	15.0 (6.1)	15.2 (6.0)
Plasma vitellin P (mg./100 ml.)	0.4 (0.5)	7.5 (6.5)	18.0 (0.5)	56.0 (0.5)	0.9 (0.4)	14.4 (0.4)	29.2 (0.4)	60.0 (0.4)
Plasma phospholipid P (mg./100 ml.)	7.7 (7.3)	16.7 (7.3)	71.4 (6.9)	164.0 (7.7)	8.8 (6.6)	26.0 (6.4)	85.6 (7.6)	135.0 (5.1)
Serum riboflavin (μg./ml.)	0.09	0.26	0.53	2.18	0.13	0.47	0.93	1.98
Liver: Total weight (g.)	29.4	37.3	49.3	54.4	31.3	42.7	49.7	50.7
Dry matter (%)	27.4	31.2	31.1	30.1	27.1	39.4	36.6	29.8
Crude protein (%)	19.3	17.4	17.2	16.0	19.6	16.5	15.8	17.6
Fat (%)	4.28	10.26	11.36	10.36	4.28	20.60	18.18	10.12
Riboflavin (μg./g.)	32.1	26.2	19.1	19.8	27.4	23.3	17.4	21.3
Serum citric acid (mg./100 ml.)	5.6	4.0	3.4	2.2	4.6	2.8	2.8	1.6

\* Figures in parenthesis are values from analyses made before starting the hormone treatment.

† These figures for plasma acid-soluble P are reported as determined. Taken in conjunction with the corresponding data for inorganic P, they suggest a large increase in ester P in the case of these two pullets, but we are not satisfied as to the validity of this large increase, which we have not observed in other similar experimental groups.

plasma and cells, as in Table 2, then the resulting figures strongly suggest that oestrogen treatments did not appreciably affect total cell volume, and that the observed increases in blood volume are mainly, if not entirely, due to an increase in plasma volume under the influence of oestrogen.

Table 2. *Effect of gonadal hormones on blood cell volume and plasma volume of the fowl*

	Pullet 53	Pullet 54	Pullet 55	Pullet 56	Pullet 57	Pullet 58	Pullet 59	Pullet 60
Oestradiol dipropionate (mg.)	0	6 × 1	6 × 2	6 × 4	0	6 × 1	6 × 2	6 × 4
Testosterone propionate (mg.)	0	0	0	0	6 × 0.75	6 × 0.75	6 × 0.75	6 × 0.75
Per pullet: Plasma (ml.)	32.4	36.8	37.6	59.9	27.6	38.3	38.2	48.0
Cells (ml.)	13.8	13.6	12.2	14.6	14.9	17.2	15.1	15.2
Per kg. live weight: Plasma (ml.)	23.8	26.5	28.7	41.9	19.2	26.8	25.6	32.4
Cells (ml.)	10.2	9.8	9.3	10.2	10.3	12.0	10.1	10.3

The data for the composition by weight of the livers have also been calculated per pullet and per kg. live weight, and are set out in Table 3. From these figures it is seen that oestrogen not only increases total liver weight and liver fat, but that it also increases total liver protein. In this experiment the increases of liver fat in the

Table 3. *Effect of gonadal hormones on composition of liver of the fowl*

	Pullet 53	Pullet 54	Pullet 55	Pullet 56	Pullet 57	Pullet 58	Pullet 59	Pullet 60
Dosage of oestradiol dipropionate (mg.)	Nil	6 × 1.0	6 × 2.0	6 × 4.0	Nil	6 × 1.0	6 × 2.0	6 × 4.0
Dosage of testosterone propionate (mg.)	Nil	Nil	Nil	Nil	6 × 0.75	6 × 0.75	6 × 0.75	6 × 0.75
Per pullet:								
Liver (g.)	29.4	37.3	49.3	54.4	31.3	42.7	49.7	50.7
Liver dry matter (g.)	8.05	11.64	15.32	16.38	8.48	16.81	18.19	15.10
Liver protein (g.)	5.08	6.49	8.48	8.69	6.12	7.06	7.82	8.93
Liver fat (g.)	1.26	3.83	5.60	5.63	1.34	8.79	9.03	5.13
Total liver riboflavin (μg.)	944	978	941	1077	857	995	864	1080
Total serum riboflavin (μg.)	2.9	9.6	20	131	3.6	18	36	95
Per kg. live weight:								
Liver wt. (g.)	21.6	26.8	37.7	38.0	21.7	29.8	35.7	34.3
Liver dry matter (g.)	5.92	8.36	11.72	11.42	5.88	11.74	13.07	10.22
Liver protein (g.)	4.17	4.66	6.49	6.07	4.24	4.93	5.62	6.03
Liver fat (g.)	0.92	2.75	4.28	3.94	0.93	6.14	6.49	3.47
Total liver riboflavin (μg.)	694	704	718	752	595	695	622	729
Total serum riboflavin (μg.)	2.1	6.9	15	91	2.5	13	24	64

oestrogenized pullets were unusually great, and naked eye inspection of the livers suggested fatty infiltration. These results are in accord with Clavert's [1944] observation that oestradiol benzoate injections increase the weight of the pigeon's liver, and that these increases involve both hyperplasia and hypertrophy. It is highly probable that the normal hypertrophy of the hen's liver during the laying cycle observed by Gericke [1945-6] also involves an increase in protein content as well as in water and fat, and that these changes are an expression of endogenous oestrogen activity in the laying fowl.

The relation of the increase in protein content to the secretion of phosphoprotein obviously calls for investigation. In both bird and mammal phosphoprotein is clearly of special significance for the nutrition of the offspring. In the mammal the phosphoprotein concerned (caseinogen) is evidently formed in the mammae and not transported thither in the blood. In the case of the bird it seems likely that the phosphoprotein concerned is formed elsewhere than in the ovary and transported thither in the blood for deposition in the yolk. If phosphoprotein-synthesizing tissue elements are to be sought in the mamma of the mammal, it seems likely that the liver is the place to look for them in the bird. Some interesting considerations of comparative biochemistry are involved; the possible effects of oestrogen on the blood proteins of the monotremes is a case in point.

As regards the effects of oestrogen on liver riboflavin, it is evident from Table 3 that the total riboflavin content was not decreased but tended, if anything, to increase. When the data for liver riboflavin are considered in relation to the total serum riboflavin, it seems unlikely that the extra riboflavin in the serum of pullets 56 and 60 can be ascribed to mobilization and withdrawal of liver reserves. This general picture is not appreciably altered when the figures are calculated on the basis of live weight instead of per pullet. In this regard, therefore, the results furnish additional support for the view that the source of the extra riboflavin in the serum of the laying or oestrogenized bird must be sought elsewhere than in the liver [Common *et al.* 1947].

Table 4. *Effect of gonadal hormones on the riboflavin content of the magnum and shell gland of the fowl*

	Pullet 53	Pullet 54	Pullet 55	Pullet 56	Pullet 57	Pullet 58	Pullet 59	Pullet 60
Dosage of oestradiol dipropionate (mg.)	Nil	6 × 1.0	6 × 2.0	6 × 4.0	Nil	6 × 1.0	6 × 2.0	6 × 4.0
Dosage of testosterone propionate (mg.)	Nil	Nil	Nil	Nil	6 × 0.75	6 × 0.75	6 × 0.75	6 × 0.75
Magnum:								
Weight (g.)	—	1.67	2.55	4.07	—	8.37	8.22	6.75
Riboflavin (μg./g.)	—	4.2	3.9	3.6	—	4.0	4.6	4.4
Riboflavin (μg.)	—	7.0	10.0	14.6	—	33.4	37.8	29.7
Shell gland:								
Weight (g.)	—	2.27	3.42	4.20	—	5.40	6.20	5.02
Riboflavin (μg./g.)	—	3.7	3.4	2.9	—	3.1	2.9	3.3
Riboflavin (μg.)	—	8.9	11.6	12.2	—	16.7	18.0	16.6
Total riboflavin of magnum plus shell gland (μg.)	2	15.9	21.6	26.8	2	50.1	55.8	46.3

The riboflavin assays on the magna and shell glands are set out in Table 4. They do not reveal any unusually high concentrations in these tissues. It does not seem likely, therefore, that the oviduct accumulates any appreciable reserves of riboflavin in advance of egg laying. At the same time, the functional magnum might show transient increases of riboflavin content in association with the transient increases in protein known to occur in association with albumen secretion [Conrad & Scott, 1942].

The investigation of the citric acid content of the sera was prompted by two considerations. In the first place, Dickens's [1941] demonstration that citrate is a major

constituent of bone has raised the question of the relation of citric acid metabolism to calcium metabolism; Dickens himself pointed out that appreciable traces are present in the hen's egg shell, and that citric acid might be concerned in the transport of calcium to the embryo. In the second place, it seems that citric acid metabolism is related to oestrogen activity, for oestradiol benzoate increases citrate excretion in women [Shorr, Bernheim & Taussky, 1942]. Although the main forms in which calcium is carried in the blood of the bird are fairly well established [McDonald & Riddle, 1945], it seemed possible that blood citrate might be increased in association with the enormous hypercalcaemia of the heavily oestrogenized bird. It will be seen from Table 1 that, in the event, serum citric acid concentration was decreased by oestrogen treatment and that there was probably also an absolute decrease. It is remarkable that this is so, since the concentrations of most serum constituents which have been investigated are increased by oestrogen rather than decreased.

#### *Experiment 8.*

Fleischmann & Fried [1945] demonstrated that thyroxine injections greatly reduce the effects of oestrogen on serum calcium in the fowl, although the thyroxine injections did not reduce the effect of oestrogen in producing hypertrophy of the oviduct. We have already discussed the possibility that serum calcium levels may normally be affected by thyroid activity as well as by oestrogen in the sexually mature pullet. Since oestrogen also raises serum riboflavin concentration, it is of interest to ascertain whether concurrent administration of thyroxine affects serum riboflavin in the same sense as it affects serum calcium, or whether, as in the case of the oviduct, it is without apparent effect.

Accordingly, eight White Wyandotte pullets of the same strain as those of Exp. 7, and all derived from the same hatching, were selected and placed under similar experimental conditions. All the birds received a total of 18 mg. of oestradiol dipropionate plus 4.25 mg. of testosterone propionate by intramuscular injection of six equal doses given on alternate days. At the same time as these injections were given, intravenous injections of thyroxine sodium (B.D.H.) were administered as indicated in Table 5.

The birds were killed on the third day after the last injection, and analyses of the blood and liver were made as in Exp. 7.

It is clear from the results (Table 5) that the thyroxine injections affected serum riboflavin level in the same sense as they affected serum calcium, i.e. the increases evoked by oestrogen plus androgen were considerably less when thyroxine was given at the same time. The degree of hypertrophy of the oviduct was not apparently affected by the thyroxine treatment, an observation in agreement with those of Fleischmann & Fried [1945].

When the data for blood volume are calculated (Table 6) in the same way as in Exp. 7, it is seen that thyroxine did not exert any appreciable effect on cell volume, but that it reduced the effect of oestrogen in increasing plasma volume.

The data for the livers have been recalculated, as in Exp. 7, and the resulting figures are given in Table 7.

On comparing the data for pullets S1 and S5 (Table 7) with those for pullets 56 and 60 (Table 3) it will be noticed that the gonadal hormone treatments in this experiment

Table 5. *Influence of thyroxine on blood and liver changes evoked by gonadal hormones*

	Pullet 81	Pullet 85	Pullet 82	Pullet 86	Pullet 83	Pullet 87	Pullet 84	Pullet 88
Dosage of oestradiol dipropionate (mg.)	6 x 3	6 x 3	6 x 3	6 x 3	6 x 3	6 x 3	6 x 3	6 x 3
Dosage of testosterone propionate (mg.)	6 x 0.75	6 x 0.75	6 x 0.75	6 x 0.75	6 x 0.75	6 x 0.75	6 x 0.75	6 x 0.75
Dosage of thyroxine sodium B.D.H. (mg.)	Nil	Nil	6 x 1.0	6 x 1.0	6 x 2.0	6 x 2.0	6 x 4.0	6 x 4.0
Live weight, initial (kg.) (age 75 days)	1.08	0.92	0.88	0.93	0.92	0.99	0.90	0.87
Live weight, final (kg.) (age 88 days)	1.31	1.27	1.21	1.24	1.17	1.27	—	1.11
Ovary: State	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent
Weight (g.)	0.27	0.23	0.24	0.29	0.24	0.29	—	0.26
Oviduct: Weight (g.)	15.8	16.4	14.2	18.4	14.3	17.5	—	17.1
Crude blood: Volume (ml.)	58.7	55.3	44.2	48.7	45.6	40.5	—	36.5
Haematocrit value	21.0	21.5	26.0	26.3	27.4	29.3	—	33.0
Serum Ca (mg./100 ml.)	98.5	114.7	83.9	74.0	60.1	41.1	—	29.7
Plasma total acid-soluble P (mg./100 ml.)	19.0	21.0	15.5	14.5	11.0	10.0	—	10.0
Plasma vitellin P (mg./100 ml.)	55	63	61	47	30	19	—	15
Plasma phospholipid P (mg./100 ml.)	106	128	109	87	47	32	—	16
Serum riboflavin (µg./ml.)	3.68	4.77	2.43	2.14	1.63	1.20	—	0.57
Liver: Total weight (g.)	37.40	34.7	34.25	20.87	32.44	32.14	—	24.60
Dry matter (%)	29.19	28.82	28.35	27.43	27.40	25.97	—	27.71
Crude protein (%)	19.27	18.74	19.01	20.35	20.40	19.86	—	21.26
Fat (%)	4.46	5.48	5.71	4.51	4.49	3.39	—	3.41
Riboflavin (µg./g.)	22.0	14.8	118.4	17.2	21.8	19.8	—	18.5
Ca intake (g.)	9.54	9.54	9.54	9.54	9.54	9.54	3.76	9.32
Ca retention (g.)	5.29	5.26	5.12	5.06	5.36	3.91	1.87	4.97
Ca retention (as % of intake)	55.5	55.2	53.7	53.1	56.2	41.0	49.8	53.3
Ca retention (g./kg.)	4.03	4.14	4.23	4.08	4.58	3.08	—	4.48

*Note.* Pullet 84 died sometime during the sixth day of experiment, having received three injections. The daily curve of calcium retention followed that for no. 88 closely so far as it went.

Table 6. *Effect of thyroxine on blood volume changes produced by gonadal hormones*

	Pullet 81	Pullet 85	Pullet 82	Pullet 86	Pullet 83	Pullet 87	Pullet 84	Pullet 88
Dosage of oestradiol dipropionate (mg.)	6×3	6×3	6×3	6×3	6×3	6×3	6×3	6×3
Dosage of testosterone propionate (mg.)	6×0.75	6×0.75	6×0.75	6×0.75	6×0.75	6×0.75	6×0.75	6×0.75
Dosage of thyroxine sodium (mg.)	Nil	Nil	6×1.0	6×1.0	6×2.0	6×2.0	6×4.0	6×4.0
Per pullet: Plasma (ml.)	46.4	43.4	32.7	35.9	33.1	28.5	—	24.5
Cells (ml.)	12.3	11.9	11.5	12.8	12.5	12.0	—	12.0
Per kg. live weight: Plasma (ml.)	35.4	34.2	27.0	28.9	28.5	22.4	—	22.1
Cells (ml.)	9.4	9.4	9.5	10.3	10.7	9.5	—	10.8

Table 7. *Influence of thyroxine on the changes in liver composition evoked by gonadal hormones*

	Pullet 81	Pullet 85	Pullet 82	Pullet 86	Pullet 83	Pullet 87	Pullet 84	Pullet 88
Dosage of oestradiol dipropionate (mg.)	6×3	6×3	6×3	6×3	6×3	6×3	6×3	6×3
Dosage of testosterone propionate (mg.)	6×0.75	6×0.75	6×0.75	6×0.75	6×0.75	6×0.75	6×0.75	6×0.75
Dosage of thyroxine sodium (mg.)	Nil	Nil	6×1.0	6×1.0	6×2.0	6×2.0	6×4.0	6×4.0
Per pullet:								
Liver wt. (g.)	37.4	34.7	34.3	29.9	32.4	32.1	—	24.6
Liver dry matter (g.)	10.91	10.00	9.78	8.21	8.89	8.35	—	6.82
Liver crude protein (g.)	7.21	6.50	6.55	6.08	6.62	6.38	—	5.24
Liver fat (g.)	1.66	1.90	1.95	1.35	1.46	1.09	—	0.84
Liver riboflavin (μg.)	823	513	634	514	707	637	—	455
Per kg. live weight:								
Liver (g.)	28.6	27.3	28.3	24.1	27.7	25.3	—	22.2
Liver dry matter (g.)	8.33	7.88	8.09	6.62	7.60	6.58	—	6.15
Liver crude protein (g.)	5.64	5.12	5.42	4.90	5.66	5.03	—	4.71
Liver fat (g.)	1.27	1.50	1.61	1.09	1.25	0.88	—	0.76
Liver riboflavin (μg.)	628	404	524	415	605	502	—	410

did not of themselves lead to as great an increase in liver fat as in Exp. 7. Simultaneous injections of thyroxine tended to give a lower fat content and lighter liver. The riboflavin contents of the livers in this experiment also appear to be somewhat lower than is usual. Further work is necessary on this aspect of the balance between the thyroid and gonadal hormones.

Mr David Luke, M.R.C.V.S., of the Veterinary Research Division, Ministry of Agriculture for Northern Ireland, kindly examined pullet 84, which died suddenly on the second day following the third thyroxine injection. Mr Luke drew our attention to the greatly enlarged heart, no other abnormality being observed *post mortem*. It was subsequently noted *post mortem* that the heart of pullet 88 was somewhat enlarged, this pullet having received a similar heavy thyroxine treatment to pullet 88.

## EFFECT OF THYROXINE ON CALCIUM METABOLISM

It has been established beyond serious doubt [Common *et al.* 1948] that oestrogen injections evoke a considerable increase in the rate of retention of calcium by sexually immature pullets, provided a small androgen injection is given at the same time. Oestrogen injections by themselves do not evoke this response, although they bring about the usual hypercalcaemia.

In Exp. 7 the rates of calcium retention were raised to a high level by the gonadal hormone treatment, but the thyroxine treatments used did not appear to reduce this high rate of calcium retention, apart from the doubtful case of pullet 87, although they were clearly effective in reducing hypercalcaemia and the elevated serum riboflavin. This observation was somewhat unexpected, since it is well known that hyperthyroidism is associated with decreased retention of calcium.

## THE RELATION OF SERUM CALCIUM TO SERUM RIBOFLAVIN

It is obvious from the results discussed in this and in our previous paper that serum riboflavin and serum calcium are broadly related, both being increased by oestrogen. When the sets of results for individual experiments (see Table 8) are plotted as in Figs. 1 and 2 it will be seen that, with the exception of the results for Exp. 6, each set falls on a curve.

Table 8. *Relation between serum calcium and serum riboflavin*

	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet
Exp. 4 ...	25	26	27	28	29	30	31	32
Calcium (mg./100 ml.)	12.6	17.4	38.3	97	12.4	28.6	76	100
Riboflavin ( $\mu$ g./ml.)	Trace	0.05	0.27	1.22	Trace	0.09	0.39	1.25
	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet
Exp. 5 ...	33	34	35	36	37	38	39	40
Calcium (mg./100 ml.)	12.7	23.4	61.4	93.5	13.5	22.8	41.8	95.5
Riboflavin ( $\mu$ g./ml.)	0.03	0.62	2.65	5.32	0.17	0.07	1.45	4.94
	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet
Exp. 6 ...	45	46	47	48	49	50	51	52
Calcium (mg./100 ml.)	12.0	17.6	64.7	103.8	9.0	23.6	54.5	98.0
Riboflavin ( $\mu$ g./ml.)	0.14	0.10	2.68	2.38	0.20	0.21	0.53	3.29
	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet
Exp. 7 ...	53	54	55	56	57	58	59	60
Calcium (mg./100 ml.)	11.9	22.7	49.8	102.6	11.5	32.7	64.8	98.4
Riboflavin ( $\mu$ g./ml.)	0.09	0.26	0.53	2.18	0.13	0.47	0.93	1.98
	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet	Pullet
Exp. 8 ...	81	82	83	84	85	86	87	88
Calcium (mg./100 ml.)	98.5	83.9	60.1	—	114.7	74.0	41.1	29.7
Riboflavin ( $\mu$ g./ml.)	3.68	2.43	1.63	—	4.77	2.14	1.20	0.57

The curve for the first set (Exp. 4, pullets 25–32) lies distinctly lower than that for the second set (Exp. 5, pullets 33–40). This is probably due in part to the circumstance that the filtrates on which the first set of riboflavin assays had been made were not specially protected against photolysis. However, all reasonable precautions of this nature were observed in the case of the other four sets of assays. Of these four sets, three fall along smooth curves, while those for the remaining set (Exp. 6, pullets

45-52, Fig. 2) are relatively widely scattered, although performed by precisely the same technique and with the same precautions against photolysis.

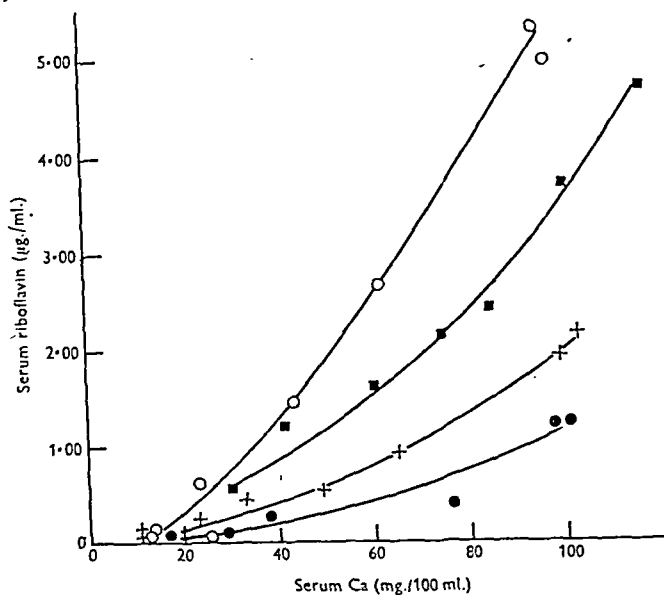


FIG. 1. Relation between values for serum calcium and serum riboflavin in Exps. 4 (●), 5 (○), 7 (+), and 8 (■).

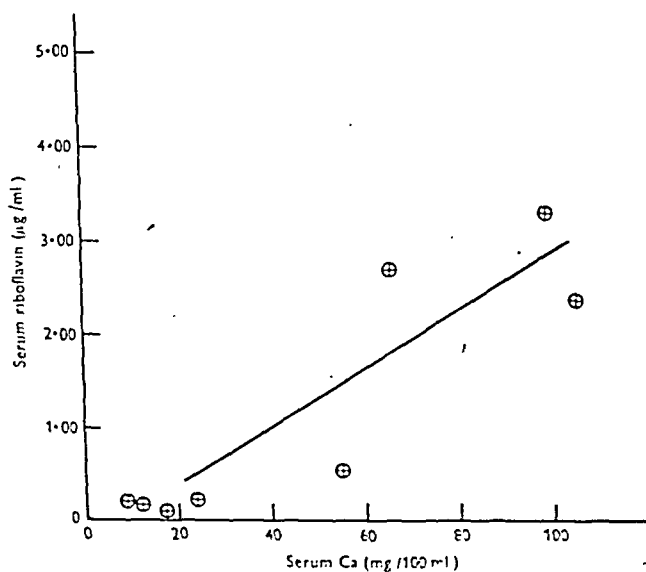


FIG. 2. Relation between values for serum calcium and serum riboflavin in Exp. 6.

Now the pullets in Exp. 6 were not of the same strain as the others, having been purchased. In fact, two of these birds went 'off their legs' while under experiment; subsequent inquiry revealed an unsatisfactory nutritional and environmental history,



and the experiment was consequently abandoned in so far as its main object (investigation of mineral metabolism) was concerned. It seems, therefore, that the wide scatter of the points for Exp. 6 is but another expression of their unsatisfactory and abnormal condition (obvious in the case of one low serum calcium value), and that the differences between the curves given by the points for Exps. 5 and 7 are real differences conditioned by differences in the totality of their endogenous endocrine balance, and possibly also their nutrition.

It is specially noteworthy that the points for Exp. 8 give a reasonably good curve, for these points were secured by the antagonistic effect of graduated thyroxine treatments on uniformly oestrogenized and androgenized pullets, whereas the other four curves were secured by graduated doses of oestrogen or of oestrogen plus androgen.

The relation of serum calcium to serum riboflavin in normal laying birds is at present under investigation. In general, the serum riboflavin values, like the serum calcium values, fall well within the range of values covered in Fig. 1, i.e. both serum calcium and serum riboflavin are lower than in the case of the more heavily oestrogenized pullets in these experiments.

#### SUMMARY

1. Treatment of the sexually immature pullet with oestradiol dipropionate leads to

- (a) an increase in liver weight;
- (b) an increase of liver fat;
- (c) an increase of liver crude protein.

These changes occur whether or not the oestrogen treatment is supplemented by androgen treatment, but they are antagonized by simultaneous intravenous injection of thyroxine sodium.

2. Treatment with oestradiol dipropionate does not appear to deplete the liver of riboflavin. The total liver riboflavin may even be increased slightly in heavily oestrogenized birds. It is unlikely that the liver constitutes the immediate source of the increased amount of riboflavin in the serum of the oestrogenized pullets.

3. Treatment with oestradiol dipropionate decreases serum citric acid.

4. The increases in serum calcium and plasma phosphorus brought about by a combined treatment with oestrogen and androgen were reduced by simultaneous intravenous administration of thyroxine sodium. However, the thyroxine treatments used did not appear to affect the hypertrophy of the oviduct or the increased rate of retention of calcium brought about by the combined oestrogen-androgen treatment.

5. The relation of serum calcium levels to serum riboflavin levels is briefly discussed.

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# CREATINE-CREATININE METABOLISM IN ADULT AND JUVENILE HYPERPITUITARISM

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The relationship of the anterior pituitary to creatine-creatinine metabolism has been studied both in animals and in man. Braier [1931] demonstrated a decrease in the creatinine excretion in hypophysectomized dogs, while Schrire & Zwarenstein [1933, 1934] produced increased elimination of creatinine in the urine of rabbits after the injection of extracts of the anterior lobe of the pituitary. Collip, Anderson & Pugsley [1934] injected rats with thyrotrophic hormone, and found an increase in the excretion of creatine in the urine. A similar creatinuria was produced in man with thyrotrophic hormone [Schrire, 1937].

The excretion of urinary creatine and creatinine has been studied in acromegaly and gigantism [Schrire, 1937; Cumings, 1944] and three features were demonstrated: creatinine is excreted in large quantities, creatine is often excreted in excessive amounts, and both these substances are eliminated in an inconstant and fluctuating manner. It was suggested that the study of the creatine metabolism in hyperpituitarism may help in the diagnosis of early acromegaly, and in assessing the state of activity of the disease.

The gonadotrophic principle of the anterior pituitary has been shown to increase creatinine excretion, and the thyrotrophic principle, acting via the thyroid gland, produces the excessive creatinuria [Schrire & Sharpey-Schafer, 1938*a*]. The same authors [1938*b*] showed that it was possible to inhibit at least one function of the anterior pituitary in acromegaly by the daily injection of large doses of oestrogen, whereby the excessive and fluctuating creatinine excretion was restored temporarily to normal. The creatinuria was not affected by these injections.

Two of the patients previously investigated by these methods have been re-examined after an interval of 8 years. On clinical grounds, the disease in both patients appeared to be no longer active. The urinary creatine and creatinine excretion has been investigated again and the results are shown and discussed below.

Recently a boy of 5½, who had been growing abnormally fast, has been under observation. Many points of similarity were noted between the boy and the two hyperpituitary cases. The history and examinations made in this boy are detailed below and all three cases are discussed in relation to anterior pituitary activity and creatine-creatinine metabolism.

## Case I

### CLINICAL OBSERVATIONS

The history of this woman (E.M.R., age 42, weight 130 lb., height 5 ft. 2 in.), up to 1937 has been previously published [Schrire & Sharpey-Schafer, 1938*a*, *b*]. At that time she was typically acromegalic. She had had a thyroidectomy performed for a

nodular goitre in 1937. Her main symptoms at that time were headaches, and she exhibited definite psychotic tendencies associated with systematized delusions. The first part of Fig. 1 shows the creatine-creatinine output in October 1937 and the response to daily injections of oestradiol benzoate. The creatinine excretion was excessive, the creatinuria was abnormal, and both substances were eliminated in an abnormally fluctuating manner. The oestrogen injections temporarily restored the creatinine excretion to normal, the creatinuria being unaffected.

She was given 51 daily injections of 10 mg. of oestradiol benzoate and discharged. Apart from a moderately prolonged withdrawal bleeding, she remained well.

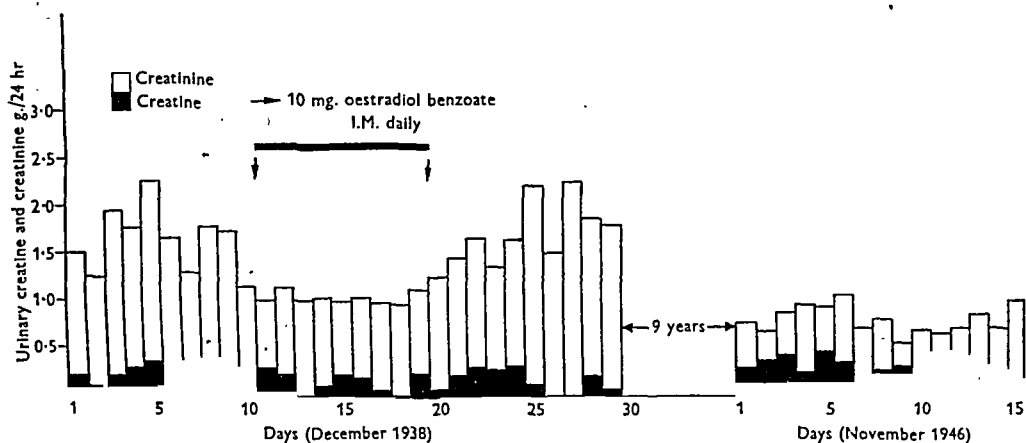


FIG. 1. Urinary excretion of creatine and creatinine in case I (E.M.R. Acromegaly).

In October 1946 the patient was re-examined. In the last 9 years there had been no ill health. Her appearance was unchanged, and she had no symptoms. Since 1937 she had not menstruated, there were occasional headaches, but otherwise she felt well.

Examination revealed that a large nodular goitre had recurred in the right lobe. Clinically she was no worse than in 1937, and her condition was judged to be stationary, or no longer active.

Fundi and fields of vision were normal. *X-ray examination* showed ballooning of the sella turcica which appeared unchanged since 1937. The mental state was unchanged. *Carbohydrate metabolism.* In 1937 there was no sugar in the urine, and the glucose tolerance test was normal. In 1946 there was daily glycosuria, and the glucose tolerance test showed a mild type of diabetes (fasting 138 mg./100 ml.—183, 212, 212, 172). *Cholesterol.* 171 mg./100 ml. (1937: 370 mg./100 ml.). *B.M.R.* Average of six estimations: +13% (1937: +26 to 12%). *17-ketosteroids.* 24 mg./24 hr.

The second part of Fig. 1 shows the creatine-creatinine excretion at this time. The creatinine is excreted in quantities normal for her weight, and does not fluctuate, and is comparable to the amounts and levels temporarily produced by the oestrogen injections. The creatinuria is still excessive and unaltered.

### Case II

The history of this man (H.A.W., age 38, weight 222 lb., height 6 ft. 6½ in.) up to 1938 has been published previously [Schrire & Sharpey-Schafer, 1938 *a, b*]. In 1938 he was a typical case of acromegalic gigantism. At that time his main symptoms were

severe bitemporal headache, and a loss of libido. X-ray examination showed a normal sella turcica.

The first part of Fig. 2 shows the urinary creatine-creatinine excretion in 1938 and the effects of daily injections of testosterone propionate. The quantity of creatinine excreted was grossly abnormal, and fluctuated in output. Creatine was not often excreted. The injection of testosterone propionate temporarily restored the creatinine to normal amounts and levels. The creatinuria was unaffected.

The patient was re-examined in April 1947. In the past 8 years health had been good, the headaches were not frequent nor as severe as in 1938. He had served 4 years in the Royal Navy (Paymaster's Branch), 2 years abroad, and had been demobilized feeling well and healthy.

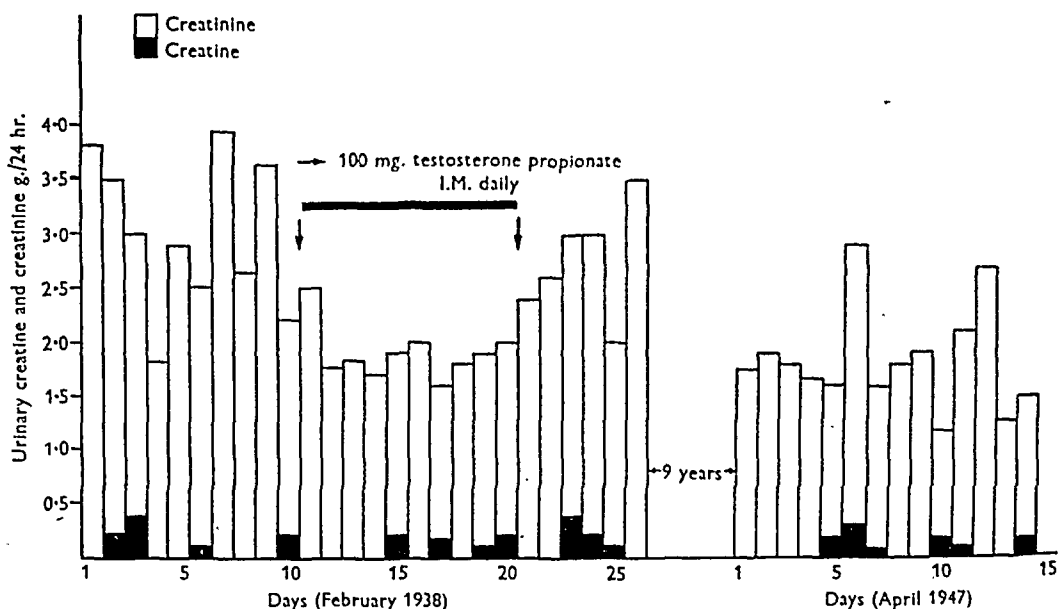


FIG. 2. Urinary excretion of creatine and creatinine in case II (H.A.W. Acromegaly and gigantism).

Hearing, vision and fundi were normal. The sella turcica was now enlarged. Libido was, and had always been, diminished, and he had occasional nocturnal emissions. He had noticed that recently physical effort tired him more easily. His complaint was judged on clinical grounds to be in an inactive or stationary phase.

The second part of Fig. 2 charts the creatine-creatinine excretion at this time. Creatinine is excreted in normal quantities and, apart from two peaks, does not fluctuate. The excretion now is comparable to the amounts and levels produced by the injection of testosterone. Creatine excretion was unchanged, and was eliminated in varying quantities from time to time.

### Case III

R.T.M. Male. Aged 5½. Born 20. iv. 41. A large child at birth, and mother had a prolonged labour. Aged 8/12 he had 10 teeth, and at 1½ years speech was established. At 2 years he was walking, the fontanelles were closed, and an X-ray of the skull showed a big skull, but a normal sella turcica.

*Family history.* Mother well. Father well but emotionally unstable. One sister of 2½—well. The boy was referred for examination by his school mistress as he had grown very





FIG. 1.

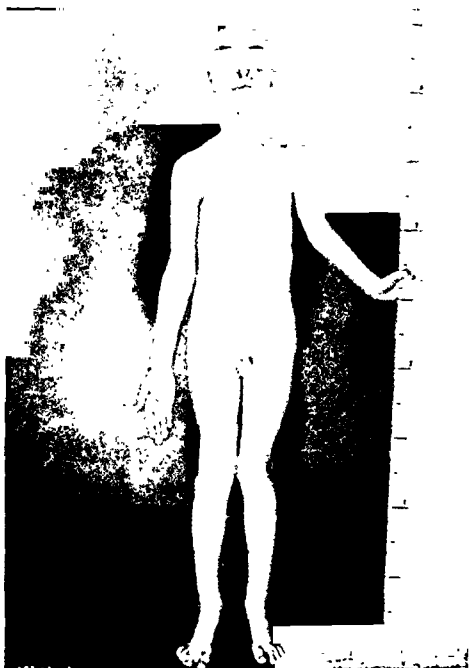


FIG. 2



FIG. 3.

Photographs of the juvenile giant (case III).

rapidly, and at school was difficult to control. He struck his smaller contemporaries, evinced a quick temper, and on occasions screamed maniacally. There was occasional enuresis. The father thought the boy in advance of his age, and clever. The school believed him to be mentally defective.

*Examination.* A big boy with a large head. There was slight prognathism. Teeth were well separated. The eyebrows were prominent and the eyes wide apart. He was very alert and, although difficult at first, he soon settled down to hospital routine and co-operated well with those whom he came to know.

*Measurements.* Standing height, 54 in. Weight, 56 lb. Crown-rump, 31½ in. Rump-ground, 22¾ in. Head circumference, 21½ in.

Eyes: fundi normal. Fields of vision (rough tests): normal. Thyroid: normal. B.P. 105/65. Heart, lungs, abdomen, central nervous system: no abnormality found. Hands: palmar creases were of simian type. Testes: both in scrotum, normal size. No evidence of precocity. Penis: ? slight enlargement. Hair distribution: normal. Glucose tolerance test: normal (124 (not fasting), 157, 109, 106, 111).

*X-ray examinations.* 13. iv. 43—straight X-ray—skull—relatively large vault. Sella turcica not unduly wide. Base rather flat.

25. x. 46. Sella turcica large—ossification of bones of hand that of a boy of 7 or 8 (age now 5½).

5. iii. 47. Stereoscopic radiograph of skull—sella of average dimensions. Clinoid processes intact. Wrists—ossification that of boy of 7 or 8.

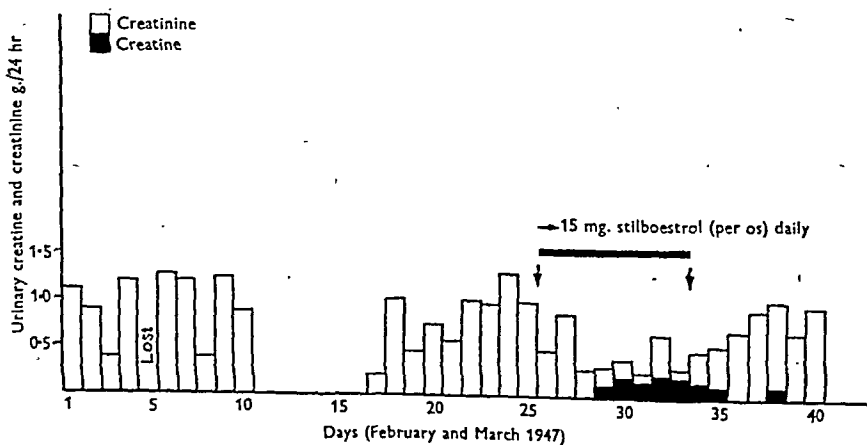


FIG. 3. Urinary excretion of creatine and creatinine in case III (R.T.M. Juvenile gigantism).

Fig. 3 shows the urinary creatine-creatinine excretion and the effects of daily oral administration of stilboestrol in this juvenile giant (cf. Plate 1). The creatinine excreted exceeded the quantities excreted by normal boys of his weight (normal = c. 0.3 g./24 hr.). The creatinine was excreted in fluctuating amounts, though this is not an abnormal finding in children. No creatine was passed, which was surprising. After ingestion of 15 mg. of stilboestrol daily, the creatinine level was restored to that normal for his weight, there was less fluctuation, and creatine appeared in the urine. After stopping the stilboestrol administration, creatine disappeared from the urine, and the creatinine returned to the fluctuating abnormally high output.



## DISCUSSION

*The adult cases (I-II)*

The new observations in cases I and II are similar, and differ strikingly from those of 8 years before. In each case the creatinine excretion had dropped to levels normal for adults of their respective weights, and the steady and constant daily output was in marked contrast to the previous abnormal fluctuations. The creatine excretion was unaltered. In case I it was still excessive, in case II it was mild, but was as seen in 1938 (Figs. 1 and 2).

The present daily excretion of creatinine in both adult patients is comparable to the amounts they eliminated during the periods of sex hormone injections. Schrire [1937] has published the results in one case of acromegaly before and after operation for excision of an eosinophil pituitary tumour. In this case, operation restored the fluctuating excessive excretion of creatinine to normal within 10 days. This was obviously fortuitous, as the exact amount of the tumour excised varies from case to case; there is seldom a complete enucleation, and the amount left behind cannot be easily assessed.

The return of the creatinine excretion to normal levels in this operated case was striking, and comparable in degree with the temporary results produced by sex hormone injections. The restoration to normal was the result of pituitary suppression. The results obtained in cases I and II, where normal outputs are now seen, thus suggest that the acromegalic process is no longer active in either case.

Of the three factors used for assessing the activity of the disease process, i.e. the increased creatinine excretion, the creatinuria, and a fluctuating output, only the first and last have been affected in the two cases studied after an interval of 8 years. The creatinuria has remained unchanged. This unaltered excretion of creatine was seen, too, in the post-operative phase of the operated case [Schrire, 1937], and also during the injection of sex hormone. An explanation of this necessitates consideration of the mode of action of the two hormones associated with these effects.

The gonadotrophic principle, or some principle inseparable from it, is believed to act directly on the creatine-creatinine processes, probably in the muscles [Schrire, 1937]. If the overactive anterior pituitary gland is inhibited, the secretion of gonadotrophic principle is suppressed and the effect on creatinine excretion is direct and immediate, for the output in the urine drops to normal.

The thyrotrophic principle, which produces the creatinuria, effects this by stimulating the thyroid gland to over-activity. Inhibition of the thyrotrophic principle by sex hormones or operation, may not of necessity affect the hyperfunctioning process in the thyroid gland which, once stimulated to overact, is independent of subsequent alterations in the activity of the pituitary gland. Case I had a partial thyroidectomy performed in 1937, but subsequently has had a recurrence, and there is at present a nodular goitre in the right lobe of the thyroid, and the B.M.R. is, on occasions, +22%. It is this recurrent toxic nodular goitre which is responsible for the creatinuria, and is unaffected by the waning pituitary activity.

This state of affairs is seen in all stages of acromegaly. Not all cases have an excessive creatinuria, e.g. case II, and some have never had creatine in the urine during the periods when they have been investigated. Similarly, alterations in carbo-

hydrate metabolism are also variable. Case II has never passed glucose in the urine, and the sugar tolerance test was normal. In 1938 case I had a normal carbohydrate metabolism, whereas in 1946 she was in a state of moderate diabetes. This suggests two possibilities: either (a) the gonadotrophic, the thyrotrophic, and the principles affecting carbohydrate metabolism are separate and independent, and of these only the gonadotrophic principle is effectively inhibited by the sex hormones; or, (b) all three hormones may be inhibited by the sex hormones, or suppressed as the disease runs its course, but the secondary manifestations produced via other endocrine glands persist as independent effects, and do not alter concomitantly with changes in the pituitary gland.

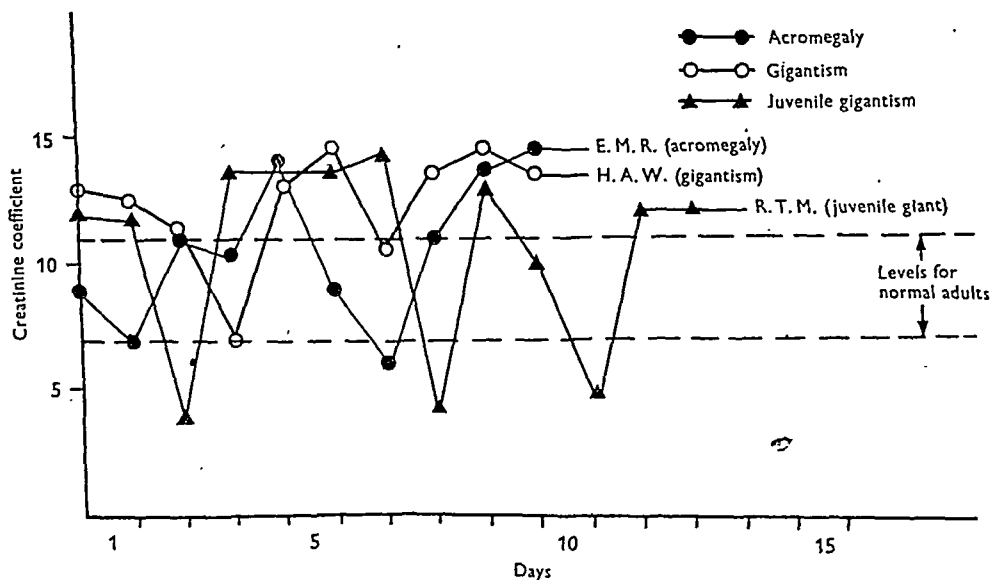


FIG. 4. Creatinine coefficients (mg. creatinine N per kg. body weight) in cases I-III.

This would explain the persistent creatinuria in case I, in the operated patient [Schrire, 1937], and during injection phase with sex hormones; and also the diabetic state in case I. Other evidence supports this latter view. Young [1937] has produced diabetes in dogs which were injected with the pituitary diabetogenic hormone. After stopping the injections, the diabetic state persisted. In acromegaly, the symptoms and signs which are produced by secondary involvement of other glands may also persist, even though the pituitary stimulus has waned or ceased. Once creatinuria and diabetes have become established, they may persist, and do not necessarily vary concomitantly with subsequent alterations in the pituitary functions.

#### *The juvenile case (III)*

Case III, the juvenile giant, showed the same excessive and fluctuating excretion (Fig. 3) as seen in the adult cases. Fig. 4 shows, for purpose of comparison, the creatinine coefficients of all three cases over a period of a few days. (The creatinine coefficient = mg. of creatinine N per kg. of body weight and varies for normal adults between 7 and 11 [Hunter, 1928].) The coefficients of all the three cases lie well beyond the accepted levels for normal adults.

Though case III excreted creatinine in excessive quantities, the fluctuation in output is not necessarily abnormal, as it is commonly seen in children. However, as the normal daily creatinine excretion for a boy of 56 lb. weight is about 0.3 g., the elimination of more than 1 g. on seven occasions in 14 days is grossly excessive. Stilboestrol ingestion reduced the high and irregular creatinine excretion to normal levels. On stopping the stilboestrol creatinine was restored to the pretherapy abnormal output. These results are comparable with those obtained with sex-hormone administration in cases I and II and in other cases previously investigated [Schrire & Sharpey-Schafer, 1938].

In the boy, before and after oestrogen administration, no creatine appeared in the urine. During the administration period, however, a brisk creatinuria occurred. The absence of creatine in the urine of a growing child is uncommon and all growing animals excrete creatine.

In case III, growth is rapid. It is suggested that in this boy all available creatine is being used for growth, and there is thus normally no surplus left for excretion as such. When the anterior pituitary has been inhibited by oestrogen, as can be seen by the diminished creatinine excretion, there is a temporary suppression of growth and thus a non-utilization of all the creatine available in the diet. Hence there is a surplus, and creatinuria occurs. When the stilboestrol is stopped, the pituitary is no longer inhibited, growth goes on, and creatine disappears from the urine.

The sella turcica in case III appears to be enlarged in the straight radiograph. Stereoscopic views do not confirm this, although in these plates the sella is certainly in the higher limits of normal. A large sella is not always found in gigantism and it is not uncommon to find a sella of normal dimensions, as is seen in case II. Gigantism does not usually become obvious early in life, though a case has been described where, at the age of 7, the patient was 6 in. taller than the average for this age [Werner, Spector, Vitt, Ross & Anderson, 1942]. The two types of pathological gigantism, eunuchoid and anterior pituitary, may be distinguished by the differences in the measurement of the crown-rump and rump-ground distances. These measurements in the boy show that the gigantism is not eunuchoid, but is probably due to a primarily over-active anterior pituitary.

All three cases present many points of similarity, clinically and biochemically. Case III conforms thus to the diagnosis of a juvenile giant due to overactivity of the anterior lobe of the pituitary gland.

#### SUMMARY

1. Two cases of acromegaly have been re-examined after a period of 8 years and the creatine-creatinine metabolism has been investigated.
2. Whereas previously the creatinine was excreted in abnormal and fluctuating quantities, now the excretion is constant and normal for their respective weights. This suggests cessation of the disease process.
3. A boy of 5½ who has grown rapidly has been investigated. The creatinine metabolism is comparable with that in acromegalic gigantism. The creatinine excretion is excessive and irregular, and is restored to normal levels by oestrogen.
4. It is suggested that the boy is a case of juvenile gigantism of pituitary origin.

The three cases were admitted to the Hammersmith Hospital under the care of Dr E. P. Sharpey-Schafer, whom I wish to thank for his help and co-operation.

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# ADRENALECTOMY AND REPLACEMENT THERAPY IN LACTATING RATS

## 5. THE EFFECT OF ADRENALECTOMY ON LACTATION STUDIED IN PAIR-FED RATS

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In previous studies of the effects of adrenalectomy on lactation in the rat [Folley & Cowie, 1944; Cowie & Folley, 1947*a, b*] we have shown, in agreement with others [Gaunt, 1941; Gaunt, Eversole & Kendall, 1942], that the operation causes a serious secretory decline but not the complete abolition of lactation. As is well known [see Ingle, 1944; Young, 1945], adrenalectomized animals, at any rate in the absence of salt therapy, may show a reduction in appetite, and since a diminution in food intake would adversely affect lactation, it is pertinent to inquire how far the lactational decline following adrenalectomy is due to anorexia, and how far to the disruption of some more direct functional relationship between the adrenals and mammary glands. We have attempted to answer this question by means of the paired-feeding technique. The experiments described in this paper give a partial answer to this question, but at the same time they serve to illustrate a defect inherent in the paired-feeding technique which in many cases may make interpretation of results obtained by this method far from easy.

This complication arises from the fact that the metabolism of an intact animal on a restricted diet may differ from that of a similar animal, the appetite of which is impaired because of the removal of an endocrine gland, not only in respect of the primary metabolic consequences of the difference between the endocrine systems of the two animals, but also in respect of the reaction of the intact animal to the changed dietary conditions. Thus the attempt to eliminate one extraneous factor, the difference in food intake, from the experimental situation may be liable to introduce further ones. This, as Ingle [1947] has pointed out, applies particularly to paired-feeding experiments on hypophysectomized or, as the results described in this paper emphasize, adrenalectomized animals, which exhibit minimal voluntary activity in contrast with pair-fed, intact controls the voluntary activity of which is often greatly enhanced in consequence of restriction of the diet.

### EXPERIMENTAL

The rats (uniparous females approximately 5 months old) and the general procedure used by us in lactation studies have been described in previous papers [Cowie & Folley, 1947*a, b, c*]. The diet was as given to the stock rats in this colony except that liquid milk *ad lib.* was not given but, instead, dried whole milk was mixed with the stock diet in the proportion 375:1000 as in a previous experiment, involving the feeding of a high-protein diet [Cowie & Folley, 1947*b*]. Supplements of raw liver and

vegetables (carrot and lettuce) in small amounts were fed as usual since these could be neglected in considering the total caloric intake. In the first of the two experiments the composition of the stock diet was as before [see Cowie & Folley, 1947*b*], while in the second a slightly modified stock diet, in which ten of the aliquot parts of whole wheat were replaced by wheat germ, was used. This change was made for the whole colony for reasons unconnected with this work.

Adrenalectomy, or in the case of the controls a sham operation, was performed on the 4th day of lactation. Each rat and her litter were weighed daily from parturition until the experiment was ended on the 17th day of lactation. Feeding of the mixture of stock diet and dried milk was begun at parturition, the mixture being offered *ad lib.* until the day of operation, and the daily food intake measured. During this period rats which persistently scattered their food, thus preventing accurate determination of the amount eaten, were eliminated from the experiment.

Each adrenalectomized rat was paired with a sham-operated partner which had littered (and had therefore been operated upon), in most cases 1 day, or in a few cases 2 or more days, later. Each pair was made up of rats which had shown, during the pre-operative period, as nearly as possible the same appetite, and whose litters were closely alike as regards birth weight and subsequent growth rate. The adrenalectomized member of a given pair was each day given food in excess of its anticipated requirements, and the amount uneaten weighed 24 hr. later; its sham-operated partner, on the corresponding day of lactation, was given the same amount of food.

In one or two cases in which the appetite of the adrenalectomized rat declined to such an extent that its pair-fed control began to suffer a serious loss of weight, the experiment ended on the 15th day to avoid exposing the intact control to unnecessary hardship. For this reason the body-weight changes and food intake figures in Table 2 are given from the 4th to the 15th day.

This paper deals with two paired-feeding experiments, in the first of which the adrenalectomized rats were given tap water to drink *ad lib.*, while in the second the tap water was replaced by 1% NaCl.

#### RESULTS

The mean growth curves for the combined litters of the adrenalectomized rats receiving no salt therapy and for their pair-fed controls are shown in Fig. 1, together with a curve for a group of sham-operated rats on unlimited food intake, which was run at the same time for comparison. Litter-growth indices [Cowie & Folley, 1947*c*] and data relating to the growth and survival rates of the litters are given in Table 1. Data relating to changes in body weight of the mothers during the experiment and to food intake are given in Table 2.

It will be seen from the curves that though restriction of the food intake of intact rats considerably reduced their lactational performance, they still lactated definitely better than adrenalectomized rats. This is confirmed by the growth and survival data for the litters, particularly the litter-growth indices, given in Table 1. Thus, while the litter-growth index for pair-fed controls was 57.5% of that for controls receiving unlimited food, the index for adrenalectomized rats was only 41.8% of this control value.

The data for the body-weight changes of the mothers from the day of operation to the 15th day (Table 2) are of interest, since they show that the adrenalectomized rats

more or less maintained their weight over this period, while the pair-fed controls, on the other hand, suffered a loss in weight of 19.7 % during this period. In this connexion it may be noted that throughout the experiment the voluntary activity of the pair-fed controls was markedly greater than that either of the adrenalectomized rats, which exhibited very little activity at all, or of the intact controls given unlimited food. This was obvious, for example, during the daily weighing when our routine procedure is, after removing a given mother and her litter from their cage, to confine the mother in a wooden box fitted with a hinged lid, while the litter is being weighed. Usually the

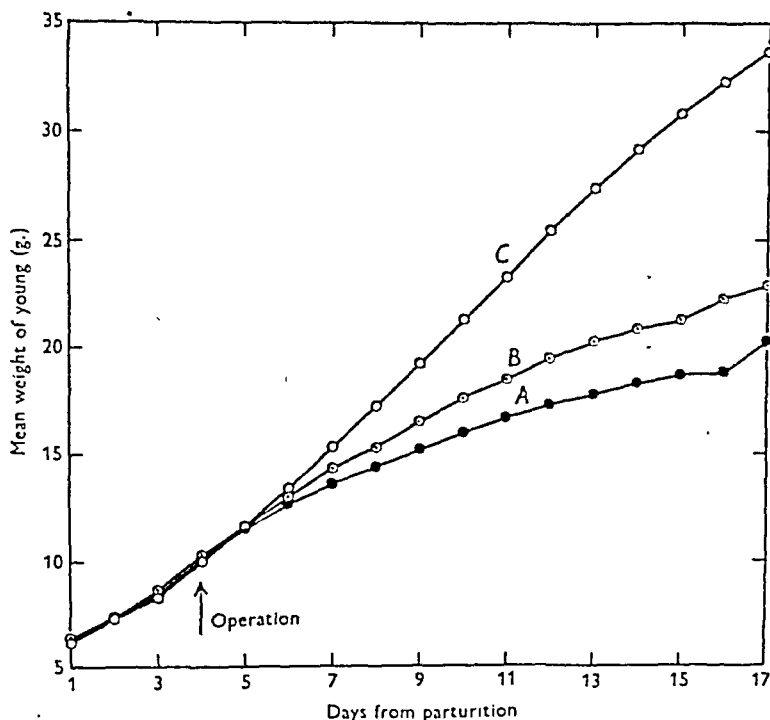


FIG. 1. Mean growth curves of the litters of groups of fifteen rats adrenalectomized on the 4th day of lactation (curve A), fifteen sham-operated on the 4th day of lactation and pair-fed with the adrenalectomized rats (curve B), and eight sham-operated on the 4th day and thereafter continued on an unlimited diet (curve C).

rats remain perfectly quiet in the box, but in the case of the pair-fed controls in these experiments, their determined efforts to escape needed in almost every case the restraint of a suitable weight placed on the lid.

The mean litter-growth curves for the second experiment, in which the adrenalectomized rats received salt therapy, are given in Fig. 2. On this occasion no rats were available to form a comparison group of sham-operated animals receiving an unlimited diet. As before, the litter-growth indices and growth and survival data for the litters are given in Table 1 and the changes in the body weight of the mothers and the food-consumption data in Table 2. It will be seen that the mean daily food consumption, calculated over the period from the 4th to the 15th day, of adrenalectomized rats receiving saline was significantly greater than that recorded in the previous experiment when no saline was given. Furthermore, these adrenalectomized animals actually gained weight during the post-operative period. It is therefore not surprising to find that the litter-growth index indicated a slightly better lactational performance

by adrenalectomized rats when salt was given than when it was not. But it was still clearly inferior to that of pair-fed controls which, moreover, had taken advantage of the larger offerings of food which enabled them to lactate better and with a smaller concomitant body-weight loss than in the previous experiment. It must be remembered, however, that it is difficult to make close quantitative comparisons between lactational performances in these two experiments, because the second one did not include a group of controls on unlimited diet which would form a common base-line of comparison between the two experiments. Lactational performance, as indicated by the litter-growth index, of groups of intact rats in this colony varies somewhat from time to time [Cowie & Folley, 1947*a*] so that, as we have pointed out before [Cowie & Folley, 1947*c*], it is desirable that a group of sham-operated controls should be included in each experiment whenever possible.

Table 1. *Data relating to the growth and survival of the litters of adrenalectomized and pair-fed, sham-operated rats*

Exp.	Treatment	No. of mothers	Total no. of young on 4th day		Mean weight (g.) of young on day		Percentage of young alive on day		Mean litter-growth index* (g./day) with S.E.	
			♂	♀	4	17	4	17		
1	Sham operation (fed <i>ad lib.</i> )	8	29	33	10.0	33.7	98	97	15.3 ± 0.7	—
	Sham operation (pair-fed)	15	60	59	10.3	22.9†	99	84†	8.8 ± 0.6	} <i>P</i> < 0.01†
	Adrenalectomy	15	62	58	10.2	20.3†	100	80†	6.4 ± 0.6	
2	Sham operation (pair-fed)	9	35	36	10.3	26.3	99	94	10.4 ± 0.8	} <i>P</i> < 0.01†
	Adrenalectomy + 1.0% saline	9	33	38	10.1	22.1	100	86	7.6 ± 0.8	

\* The litter-growth index of a group of rats is defined as the mean daily gain in weight per litter over the 5-day period from the 6th to the 11th days [Cowie & Folley, 1947*c*].

† These values are for the litters of thirteen mothers only, since two pairs of rats were killed on the 15th day (see text).

‡ Calculated by the 't' test from the paired data.

Table 2. *Body-weight changes and food consumption of adrenalectomized and pair-fed, sham-operated rats*

Exp.	Treatment	Mean percentage change in weight of mothers between 4th and 15th days with S.E.	Mean daily food consumption (g./day) between days	
			6 and 11*	4 and 15
1	Sham operated (fed <i>ad lib.</i> )	+ 8.1 ± 1.2	Not measured	Not measured
	Sham operated (pair-fed)	- 19.7 ± 2.0	21.9 ± 1.1	21.1 ± 1.1
	Adrenalectomy	- 1.5 ± 1.5		
2	Sham operated (pair-fed)	- 12.9 ± 2.4	24.0 ± 1.4	24.7 ± 1.4
	Adrenalectomy + 1.0% saline	+ 7.6 ± 2.4		

\* i.e. the period over which the litter-growth index [Cowie & Folley, 1947*c*] was calculated.



## DISCUSSION

The marked difference in lactational performance between adrenalectomized and sham-operated rats, previously described [Cowie & Folley, 1947*a*], is certainly reduced when the food intake of the two types of rat is equalized. Nevertheless, the litter-growth and survival data (Table 1 and Figs. 1, 2) show that the performance of the adrenalectomized rats was significantly inferior to that of pair-fed controls, irrespective of whether or not the former received NaCl therapy. In fact, the litter-growth and survival data (Table 1) indicate that the difference in lactational performance between the two types of rat was if anything slightly enhanced when the

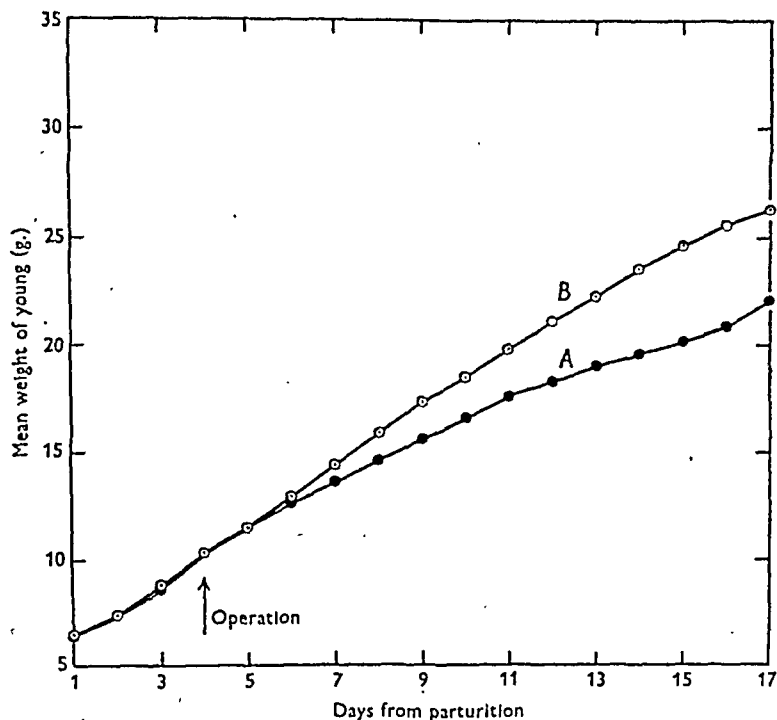


FIG. 2. Mean growth curves of the litters of groups of nine rats adrenalectomized on the 4th day of lactation and thereafter given 1% NaCl instead of water to drink (curve A), and nine sham-operated on the 4th day of lactation and thereafter pair-fed with the adrenalectomized rats (curve B).

adrenalectomized animals received NaCl. This was probably because the NaCl therapy improved the appetites of the adrenalectomized rats, which meant that their partners received more food than the pair-fed controls in the other experiment, an increment which they seemingly proceeded to convert into milk more efficiently than their adrenalectomized pair-mates. It must be admitted that the difference between the milk yields of adrenalectomized and pair-fed rats, though significant, was relatively slight in both experiments; and at first sight it might appear that post-operative anorexia could account for most of the lactational decline following adrenalectomy and that the effect attributable to the interruption of a more direct relationship between the adrenals and the mammae is relatively insignificant.

However, the present experiments well illustrate the fact that adrenalectomized rats and pair-fed controls are not strictly comparable for studies on milk secretion, since the controls in both experiments lost considerable weight during the experiment

while the body weight of the adrenalectomized animals changed very little, or, when NaCl was administered, increased by about the same percentage as that of intact controls fed *ad lib*. These findings become intelligible in the light of the fact that the pair-fed controls continuously exhibited quite unusual voluntary activity, exceeding that of intact rats on a normal food intake, while the activity of the adrenalectomized rats was much reduced. Thus it seems certain that the metabolic rate of the pair-fed controls was considerably greater than that of the adrenalectomized rats and probably exceeded that of the intact rats receiving food *ad lib*. Such an increase in total metabolism under conditions of limited food intake would undoubtedly result in increased tissue catabolism leading to loss in body weight.

In order quantitatively to interpret the effects of restriction of the food intake on the lactational performance of the sham-operated rats in these experiments, it is necessary to know how far milk secretion tends to be maintained under these conditions at the expense of tissue catabolism, and also the relative demands resulting from the increased voluntary activity on the food and on the body tissues. The answers to these questions are of course not known, but three possibilities are considered below in a discussion in which, when we refer to the body-weight losses suffered by the pair-fed controls, we mean the net differences between the slight body-weight increase regularly observed in our intact rats during lactation [see Folley & Cowie, 1944; Cowie & Folley, 1947*a, b*] and the striking losses observed in the pair-fed controls.

(a) The increased voluntary activity of the pair-fed controls may have occurred not only at the expense of the body tissues, the catabolism of which was reflected in the body-weight losses, but also at the expense of a proportion of the food which would otherwise have been available for milk secretion. This would imply that the mean daily amount of food used for maintenance as distinct from milk production, the 'maintenance ration' of the dairy husbandman, was somewhat greater in the pair-fed controls than in the adrenalectomized rats, and probably also than in the intact rats receiving food *ad lib*. The superiority in lactational performance of the pair-fed controls over their adrenalectomized counterparts would thus have been greater had there been no increase in voluntary activity. In other words, if the alternative we are considering were true, it could be said that the pair-fed controls lactated better than the adrenalectomized rats on a smaller 'production ration' and would have excelled them still more had their 'production ration' been equal to that of their pair-mates.

(b) The increased tissue catabolism represented by the body-weight losses of the pair-fed rats may have represented the exact cost of the enhanced metabolism resulting from their hyper-activity, in which case it may be concluded that their milk yield was very little different from what it would have been had they undergone no increase in spontaneous activity. If this were the case, it would follow that our results give a substantially true quantitative picture of the effect of adrenalectomy on lactation.

(c) The enhanced tissue catabolism and consequent body-weight losses exhibited by the pair-fed controls may have been only partly due to their increased voluntary activity, the rest being ascribable to an effort to maintain milk secretion. In this event it would follow that the pair-fed controls would have lactated hardly more, perhaps even a little less, efficiently than the adrenalectomized rats, had not the possession of adrenal glands in some way conferred upon them the power of secreting milk at the expense of tissue catabolism, an ability which according to our results

seems to be absent from adrenalectomized rats. Incidentally, in this latter connexion it may be remembered that Long, Katzin & Fry [1940] have suggested that the adrenal cortex may promote the mobilization of endogenous protein by exerting a direct effect on the conversion of tissue protein into amino acids. If this alternative were true, it seems possible that had these rats not increased their voluntary activity they might have secreted more milk than they actually did, but this would depend on how far under such conditions body tissues, which otherwise would have undergone catabolism to support increased voluntary activity, could have been diverted to the support of milk secretion.

The paired-feeding technique, as used in these experiments, does not permit of differentiating between the above possibilities (and others differing from one or the other of them only in degree), though it may be said at once that the third alternative seems *a priori* rather unlikely. As between the first and second alternatives we favour the first as being the more likely, since it seems probable that under conditions of insufficient food intake an increased metabolism due to enhanced voluntary activity would be preferentially compensated for by an increased use for maintenance of food which would otherwise be available for the support of milk secretion. This interpretation is supported by the indication from our results (Table 1) that the disparity in lactational performance between the adrenalectomized rats and their intact pair-mates was enhanced when NaCl was administered to the former with the result that not only they, but also the pair-fed controls, were able to utilize more food so that the body-weight losses of the latter were reduced.

In order to study this question adequately it would be necessary to 'pair-feed' not on a basis of total food intake, but on a basis of 'production' ration for milk secretion, a procedure involving the theoretical division of the daily ration into two moieties which is not possible on present knowledge. It is hoped, however, that further information may accrue from experiments involving the 'forced-feeding' of adrenalectomized rats which are contemplated.

The present results allow only of the conclusion that adrenalectomy does result in a significant decline in lactation, quite apart from secondary effects due to anorexia, to a degree which on the face of it is not very large but may well in actual fact be larger than the paired-feeding technique is capable of revealing.

#### SUMMARY

1. Since studies of the effect of adrenalectomy on lactation may be complicated by secondary consequences of post-operational anorexia, the question has been investigated in rats by the paired-feeding technique.

2. The difference in lactational performance between adrenalectomized and intact rats was considerably reduced when the food intake of the two types of rat was equalized, but the superiority of the pair-fed controls was still significant even if relatively slight. This conclusion holds irrespective of whether or not the adrenalectomized rats were given NaCl therapy, though in the former case there was a slight indication that the superiority of the controls is more marked.

3. Intact rats on a reduced food intake showed a marked increase in voluntary activity, while the activity of their adrenalectomized pair-mates was much reduced. The former lost considerable body weight during the experiment, while the body

weight of the adrenalectomized rats was nearly or fully maintained, or, when NaCl was given, increased.

4. It is impossible to say what effect the enhanced tissue catabolism resulting from hyper-activity had on the milk yield of the controls, but certain possibilities are discussed. It is concluded that the paired-feeding method is inadequate for quantitative studies of the effect on lactation of procedures which incidentally reduce the appetite.

We are indebted to Dr S. K. Kon for placing the facilities of his rat colony at our disposal, and to Mr S. C. Watson for technical assistance.

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# THE ACTION OF THIOURACIL UPON THE SPURS OF THE DOMESTIC FOWL\*

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Cocks and capons, which are kept upon a diet containing 0.5 % of thiouracil on a dry-weight basis, develop peculiar modifications of the spur after certain periods of treatment [Juhn, 1946]. The birds showing these developments belonged to separate experimental series, which were primarily outlined for an analysis of the range of reaction of feather pigments in pure-bred and in hybrid fowl and which were not directed toward the study of the effect as such on the spur. The descriptions given here are therefore a first report. However, experiments specifically oriented toward a study of the action of thiouracil upon the spurs of fowl, at various ages, for varying lengths of time, etc., and under varied conditions of supplementary vitamin and hormone administration, are now in progress in this laboratory under research grant No. RG-638, U.S. Public Health Service.

Routine inspection of the spurs of all birds, thiouracil-treated and others, was inaugurated following notice of the publication by Caridroit & Régnier [1944] of their observations on the changes which take place in the spurs of cocks which were thyroidectomized as adults. These authors describe cases in which the operation was fully successful and also cases where the removal of the gland was incomplete and regeneration of thyroid tissue occurred.

Several months after thyroidectomy, the spur, which in the normal adult male is cylindrical, long, pointed, and firmly attached to the tarso-metatarsal bone, became loose at the junction with the shank and its shape was now cylindrical with a blunt tip. Longitudinal sections of spurs from operated and control birds showed that the normal bony central axis was reduced and replaced by fibrous connective tissue. This fibrous connective tissue separated the remnant of the bony spur core from the shank and caused the characteristic loosening. Tissue dedifferentiation occurred in two ways: (1) at points distant from the blood vessels simple decalcification set in; (2) around the blood vessels calcified tissue was replaced by reticular tissue. Spur growth was similar in the operated and control birds and proceeded at an average rate of about 2 mm. per month.

In a number of incomplete thyroidectomies, there was no noticeable change in the spur; in others, the spur first lost its normal shape and became loose, but later these spurs again became firmly attached as other signs of thyroid deficiency also simultaneously improved.

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## OBSERVATIONS

In this laboratory, birds were examined for condition of the spurs at autopsy and during life. Records made at autopsy of twelve adult birds (nine capons and three cocks) which had not served in any experiments, showed all spurs to be firmly attached and normal in shape. About half of these birds were extremely emaciated at death, while the others were in fair to excellent flesh. Four adult cocks, which were fed sodium selenate at the rate of ten parts per million in the diet for 11 months and 21 days and then killed, also showed taut and normally shaped spurs. This was also true of two cocks earlier treated with oestrone.

Among twelve individuals first fed thiouracil and then transferred to other tests, both normal and abnormal spur conditions were found, in these cases the extent of the modification apparently depending upon the length of time which the birds previously had been kept upon thiouracil.

Autopsy records and histories of fourteen birds that had been treated only with thiouracil showed clearly that the degree of modification depended in the first place upon the age of the bird at the time administration of the drug was initiated, and secondly upon the length of time the treatment was continued. No relation appeared between the general condition of an experimental fowl recorded at autopsy and spur effect exhibited by the individual.

The most pronounced departure from the normal spur condition developed in two cocks that were continued upon a thiouracil diet from about 7 weeks of age up to their death, approximately 2 years later. At that time, the spurs of these birds were markedly loose and curved downward and around so that the spur tip pointed toward the shank bone instead of away from it. X-ray photographs showed the presence of a very small portion of the calcified core which extended from a point slightly distant from the shank bone about one-eighth to one-fourth the length of the spur. Young adults of 5-6 months showed a definite loosening after only about 2 months of treatment, but in 1-year-old birds of this group substantially longer periods of treatment were essential to effect a change. Birds over 2 years of age showed only a very slight response, or none at all, even after a year of thiouracil administration.

Flexibility and a modified shape were found to be constant characteristics of the thiouracil-modified spurs, but an additional feature was recognizable even in birds of the autopsy group. This was a faintly bluish rose zone in that part of the spur immediately adjacent to the shank bone and extending toward the spur tip; in older birds for distances of approximately 1 cm. The development of this hyperaemic zone was followed through the examination, at intervals, of twenty-eight living birds. Appearance of the zone preceded flexibility in all cases where thiouracil administration was commenced in birds with well-developed spurs firmly attached. It was even found that a faint 'blushing' could be induced in an apparently wholly unmodified spur by slight manipulation and that this response represented the first recognizable effect.

However, cockerels fed thiouracil from the day of hatching, at 6 months had well-grown spurs that were extremely loose, but these birds never showed the pinkish zone. The discrepancy between the groups is no doubt readily interpreted through the findings of Kozelka [1933, p. 73] on the origins of the spur in the normal bird. 'At time of hatching the spur of either sex is a small oval thickening of ectoderm. In the

female it normally remains a comparatively small, oval, movable structure throughout the life of the bird; in the male it continues to develop and eventually becomes attached to the shank bone by a fusion of the bony core of the spur with the outgrowth from the shank bone induced by the presence of the spur.' Removal of the spur in infantile male chicks prevents development of the outgrowth from the shank bone but the removed spur will develop when transplanted to fleshy portions of the body.

Treatment with thiouracil commenced before the normal secondary fusion of the spur to the shank bone sets in apparently inhibits this union at the prospective locus. Spur growth, however, proceeds independently in absence of the junction. This is in harmony with Kozelka's observations [1929, 1932] on the development of spurs grafted to other sites of the body such as the comb, where no bony substrate obtains.

The hyperaemic zone which is the characteristic first thiouracil effect in the adult fully attached spur, on the other hand, most probably represents a directed invasion by vascular elements of the point of junction, leading to the progressive decalcification and tissue dedifferentiation described by Caridroit & Régnier [1944].

The thiouracil effect in the spur is sometimes characterized by a certain asymmetry in its first manifestation: among the group examined during life, six birds first showed it in the right spur, two in the left spur.

The sequences developing in the wake of thiouracil administration in the spurs may be very nicely followed in serial X-ray photographs of which Pl. 1, figs. 1 and 2 are examples.

The division between the shank bone and the residual bony core of the experimental bird's spur is clear, and measurements of this distance should provide a more objective measure of effect than observations on relative degrees of flexibility, colour changes, etc.

#### SUMMARY

The modifications arising in the spurs of cockerels, cocks, and capons kept upon a diet containing 0.5 % of thiouracil are described. When administration of the drug is commenced in the adult fowl there develops a characteristic hyperaemic zone in the spur at its junction with the shank bone. This is followed by a loosening of the spur from its normal firm attachment so that it becomes movable. The spur also undergoes changes in shape. X-ray photographs demonstrate the reduction of the bony central core of the spur and decalcification at the point of union with the shank.

Spurs of cockerels fed thiouracil from 1 day of age exhibit continued growth, but the normal attachment of the spur to the shank never takes place. A hyperaemic zone is not developed here, indicating that union is inhibited in these cases while in the adult there is an active resorption of tissue at the spur-shank junction.

These findings are discussed in connexion with the observations of Caridroit & Régnier on the spurs of cocks thyroidectomized as adults, and those of Kozelka on growth and development of normal and of transplanted spurs.

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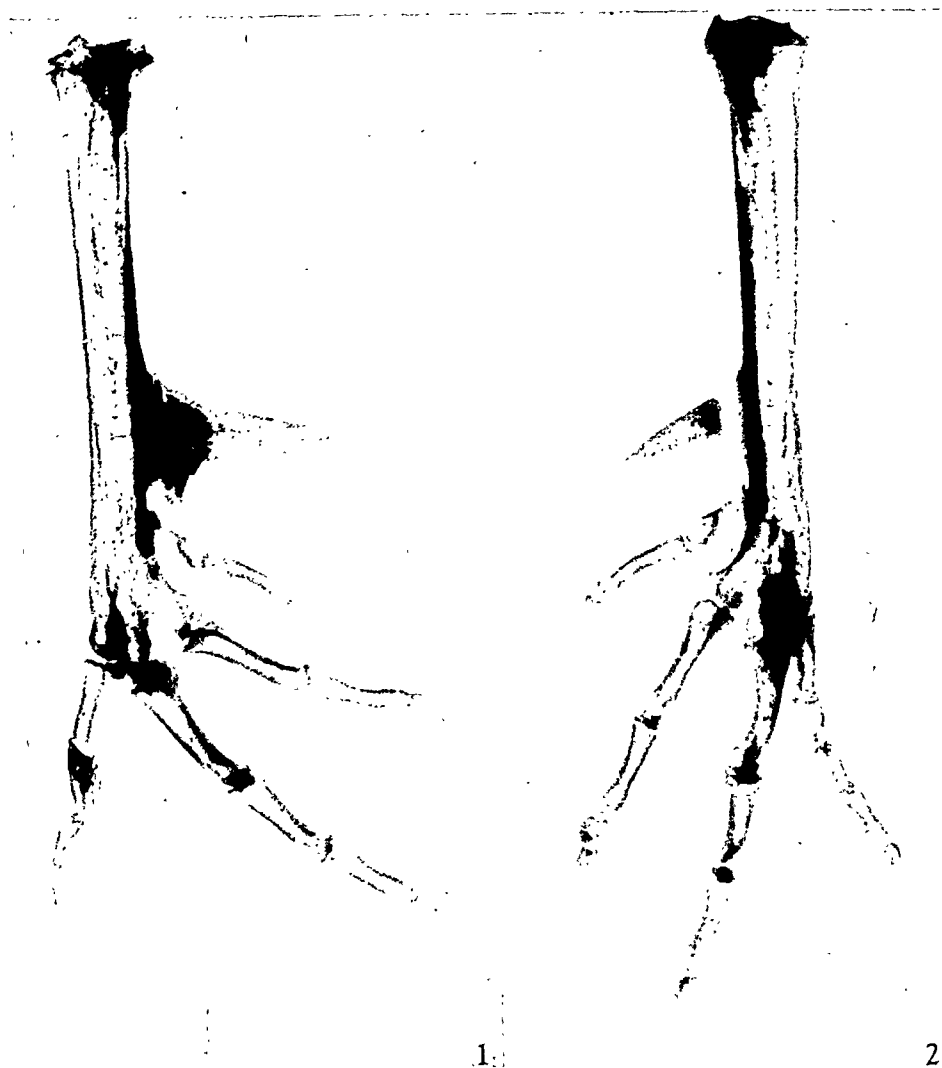


FIG. 1. X-ray photograph, prepared 30 October 1946, of the spur and shank bone of normal control cock 658, hatched 14 July 1944.

FIG. 2. X-ray photograph, prepared 30 October 1946, of cock 689, hatched 14 July 1944 and placed upon a diet containing 0.5% of thiouracil, 23 January 1945. The effect of the drug is seen in the detachment of the spur from its normal junction with the shank bone, as well as in the change of shape of the spur and in the reduction of its central bony core.





# THE EFFECT OF TESTOSTERONE PROPIONATE ON THE LIVER OF ADULT FEMALE RATS

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In view of the well-known observation that the liver appears to inactivate testosterone propionate, it may be considered justifiable to investigate whether the hormone has any morphologically observable effect on that organ.

The question whether the liver-cells undergo changes in size under the action of the testosterone has been discussed by Korenchevsky & Hall [1938]. In their experiments on male rats, the results did not show any convincing enlargement of the cells. Korenchevsky, Hall, Burbank & Cohen [1941], however, in a minor experiment on spayed female rats, found a tendency to enlargement of the liver-cells.

The effect of the hormone on the amount of liver glycogen has been studied by Gaunt, Remington & Edelman [1939], who, after histological examination, found that in three examined rats testosterone propionate produced merely a slight increase, if any, of this substance.

Certain cytological changes in the liver in connexion with the effects of androgens were observed by Korenchevsky [1941] in an investigation involving a considerable number of animals. The material consisted of 23 normal, 77 castrated, and 85 ovariectomized, adult rats, previously described by Korenchevsky, Hall & Burbank [1939]. During a period of  $3\frac{1}{2}$  months, weekly doses of 0.75 or 7.5 mg. of pure testosterone or of 0.75 or 7.5 mg. of testosterone propionate were administered. In addition, 29 normal ageing female rats were treated for 2 months with testosterone propionate, etc. Basophil granules in the liver-cells, larger in number in the males than in the females, were reduced in number and size by gonadectomy in both sexes, whilst the liver was reduced in weight. When the gonadectomized animals were treated with androgenic hormones, the weight of the liver was restored to normal, and the granules reverted to the normal number and size.

With testosterone propionate Blackman, Thomas & Howard [1944] have made experiments on  $3\frac{1}{2}$ -weeks-old dogs mainly with regard to the effect of the hormone on the liver. One male dog received during 6 weeks 420 mg. of testosterone propionate (Perandren, Ciba). Another male dog and two bitches were treated with testosterone propionate during 14 weeks with total doses of 970 mg. The increase of liver weight was 29–74 %, and of body weight only 14–18 % over that in uninjected control dogs. The authors did not find any histological change which could explain the increase of weight.

In non-castrated animals, Korenchevsky & Hall [1938] found that testosterone had no marked effect on the nine male rats which served as material for their studies. Selye [1939*a*, *b*], who studied the effect of the hormone on seven female mice, found the liver weights were approximately the same in the treated animals and in seven controls.

The question whether testosterone affects the liver weight, however, cannot be regarded as settled, in view of the smallness of the groups in these two investigations. I have accordingly taken it up in the present study.

#### MATERIAL AND TECHNIQUE

The material consisted of 75 female rats, aged about 16 months, and weighing between 175 and 255 g. They were fed with oil cakes [Gard, 1944] as well as with milk and water *ad libitum*.

After weighing, the animals were divided into three equal groups. The first group received injections of testosterone propionate dissolved in sesame oil, the second sesame oil alone, whilst the third group received no injections.

Group I received every other day for 1 month an intramuscular injection of 2 mg. of testosterone propionate, dissolved in 0.08 ml. of sesame oil (Perandren, Ciba). Each animal thus received 15 injections, and the total dose amounted to 30 mg. Group II was treated in a similar way, except that injections consisted of sesame oil alone (0.08 ml.).

After a month all the animals were weighed and killed with ether, and the liver was removed as quickly as possible and weighed. The animals were killed at the same time of the day. A small piece was taken from each liver and fixed for histological treatment and examination. The remainder of the liver was weighed and freed from water by keeping in a thermostat at an air temperature slightly above 100° C. After 5 days (120 hr.) the dry liver was weighed, and the total dry weight of the liver was estimated.

The weights were read with an accuracy within 0.0001 g.; two decimal places only, however, are shown in the tables.

The piece of liver taken for histological examination was divided into two parts; one of them was stained with hematoxylin-eosin, the other by Best's glycogen staining method.

#### RESULTS AND DISCUSSION

The liver weights were treated statistically, and the results are shown in the table.

Table 1. *Changes in liver weight in female rats injected with androgen*

Injections	No. of animals	Liver weight (g./200 g. body weight)	Fresh weight of liver (mg.) $M \pm \sigma_m$	Dry weight of liver (mg.) $M \pm \sigma_m$	Dry weight as percentage of fresh weight $M \pm \sigma_m$
Testosterone propionate in sesame oil	22	9.9	$10.91 \pm 0.40$	$2.98 \pm 0.11$	$27.4 \pm 0.34$
Sesame oil	21	10.0	$11.00 \pm 0.36$	$2.79 \pm 0.09$	$25.3 \pm 0.61$
None	22	10.3	$10.86 \pm 0.30$	$2.81 \pm 0.07$	$25.9 \pm 0.18$

An examination of the body weight at the end of the experiment showed that the hormone-treated animals were slightly heavier than those in the two other groups. The average liver weight corresponds rather closely in the three groups, and this applies also to the liver weight per 200 g. of the body weight. No effect either of the hormone or of the sesame oil can be statistically shown as regards these fresh weights.

The hormone-treated animals show a somewhat higher average dry weight than those in the control series, where the relative value for all the controls is approximately the same. The difference between the hormone-treated animals and the controls is  $0.17 \pm 0.13$  g. and thus is not statistically significant. It is more serviceable to reckon the dry weight as percentage of the fresh weight for each animal and then to estimate the mean value and its standard error. As shown by the table, the mean value for the hormone-treated animals is  $27.4 \pm 0.34\%$ , and for the animals which received sesame oil alone  $25.3 \pm 0.61\%$ . The difference is thus  $2.1 \pm 0.70\%$ , and is statistically significant. Also in a comparison between the hormone-treated and the non-treated animals a statistically significant difference, viz.  $1.5 \pm 0.39\%$ , is obtained. The hormone must thus have entailed an increase in the quotient dry-substance/water in the liver, though a relatively small one.

The histological examination showed in the testosterone-treated animals a slight thickening of the intima in the interlobular branches of the hepatic artery, but no marked pathological changes. The result corresponds in the latter respect with the observations made by Selye [1939b] in mice. In fact, this author, who had observed that oestrogens in mice cause liver necrosis or a periportal hepatitis, looked in vain for such phenomena in the mice treated with testosterone propionate.

The fact that the fresh weight had not increased as a consequence of the hormone treatment seems to gainsay the supposition that the hormone entails a hypertrophy or hyperplasia of the liver parenchyma or stroma, or of both parts.

In this connexion it should be mentioned that a comparison of the glycogen-stained sections from the three experimental groups did not provide any basis for judging whether the amount of glycogen had changed under the action of the hormone.

From a comparison of the sections stained with haematoxylin-eosin in the group treated with sesame oil alone and the non-treated group, it seems that the epithelial liver cells in the oil-injected group contain a more copious number of vacuoles resulting from the discharge of fat than the other group, which is not surprising, as the first-mentioned group had received a considerable additional supply of fat. A comparison in this respect of the sections from the group treated with hormone plus oil with those from the group treated with oil alone gave no distinct indication of any difference in the amount of fat: on this account, however, the possibility that there may have been such a difference cannot, of course, be ruled out.

Whether the increase of the dry substance had been due to a larger amount of glycogen or fat, or had been caused in some other way, is a problem which it is difficult to solve morphologically. More reliable results could presumably be obtained by quantitative chemical investigations, which, however, lie beyond the scope of this study.

#### SUMMARY

Treatment of adult female white rats with testosterone propionate for 1 month results in an increase in the ratio of dry-substance to water in the liver without an increase of its fresh weight.

The author is grateful for the valuable assistance in statistical treatment of the results given by Mr E. Lander, Actuary to the State Institution of Race Biology, Uppsala.

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## SEBACEOUS GLANDS

### 1. THE EFFECT OF SEX HORMONES ON THE SEBACEOUS GLANDS OF THE FEMALE ALBINO RAT

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That the sebaceous glands are affected by the sex hormones has been suggested by the clinical observations of Hamilton [1941], who observed the appearance of acne after administering androgens to human beings. Lawrence & Werthessen [1942] have reported successful treatment of this condition with orally administered oestrogens. Experimental work carried out by Hooker & Pfeiffer [1943] and De Graaf [1942, 1943] is referred to in the discussion.

The object of this work was to devise a technique for the exact study of the effect of various hormones on the sebaceous glands of the rat.

#### METHODS

Groups of female Wistar rats having initial body weights of about 40–60 g. were injected subcutaneously with 1 or 100  $\mu$ g. of oestradiol benzoate (Dimenformon), with testosterone propionate (Neo-Hombreol), or with 1/10 or 1/2 mg. of progesterone, each daily for about 30 days.

The rats were killed, and the skins were removed from mid-dorsal incisions, stretched on cork boards, fixed in Bouin's solution for 24 hr., and afterwards washed in running water for 48 hr. Portions of skin from the mid-dorsal region of the body were then transferred to 70% alcohol, embedded in 'ester wax' using the methods and formulae of Steedman [1945, 1947] and sectioned sagittally at 6  $\mu$ . The sections were stained in methylene blue and erythrosin and mounted in 'Sira'.

Each sebaceous gland consists of a sac-like alveolus associated with a hair follicle. The whole alveolus is full of sebaceous cells and the secretion is produced by the breakdown of these cells in the mouth or duct region where the alveolus opens into the hair follicle. The alveolus is enclosed by a basement membrane, over the whole area of which replacement of cells seems to occur. This description is contrary to the usual accounts, which state that new cells are derived only from the duct region; but it is in accordance with the view of Bullough [1946]. The terms 'gland' and 'alveolus' are used synonymously in this paper, it being assumed that the glands are simple. It is, however, possible that grouped alveoli observed in some cases may represent branched glands.

The following points were considered of possible interest in the examination of the activity of the sebaceous glands.

(1) The appearance of the glands; for example, the mitotic activity of cells in the region of the duct and basement membrane and the degree of breakdown of cells in the mouth of the alveolus.

(2) The number of glands per unit area of skin.

(3) The size of the individual glands.

The number of alveoli and the number of cells in each alveolus in a measured 5 mm. length of section were counted for each of three different sections cut from a single block of skin; for record purposes these sets of data were respectively totalled for the three sections, giving a 'Sebaceous cell count' and an 'Alveolar count' for 15 mm. of section in each case.

#### RESULTS

The results are shown in Table 1, which includes data obtained in two experiments carried out at different times (S and V series), and in Table 2 which contains the data from a third experiment with separate control animals (U series).

It is clear (Table 1) that treatment with 100  $\mu$ g. per day of oestradiol benzoate reduces considerably the total number of sebaceous cells. A comparison of the mean sebaceous cell count in the 1  $\mu$ g. per day group with that of the controls gives  $t=3.96$ , which is also highly significant ( $P<0.01$ ). Similar comparisons of the figures for alveolar counts give  $t=3.44$  with  $P<0.01$  for the 100  $\mu$ g. per day group and  $t=2.36$  with  $P$  between 0.02 and 0.05 in the 1  $\mu$ g. per day group.

In the case of the group treated with testosterone propionate, there is clearly a large significant increase in the mean sebaceous cell count. A comparison of figures for alveolar count with those of the controls shows an increase giving  $t=2.22$  and  $P$  between 0.02 and 0.05.

These changes in the sebaceous cell count may be due to changes in size or in number of the alveoli. Similarly, the alveolar count depends on the size of the alveoli as well as on their abundance.

It is possible to arrive at an interpretation by a consideration of the derived figures for the average cell count per alveolus, which are given in the last column of Table 1. A reasonable estimate of the mean may be obtained by dividing the total sebaceous cell count for the whole group by the total alveolar count for the group. For the controls this figure is 7.5 with a range in the figures for individual animals of 6.2-9.4. Corresponding figures for the 1  $\mu$ g. per day group are 6.3 with a range of 4.7-7.9; and for the 100  $\mu$ g. per day group, 3.7 with a range of 2.5-4.7. It is clear therefore that there is reduction in size of the individual alveoli in the 100  $\mu$ g. per day group and less marked reduction in the 1  $\mu$ g. per day group. A photomicrograph of a section of a normal gland is shown in Pl. 1, fig. 1 and a section of a gland from a rat treated with 100  $\mu$ g. of oestradiol benzoate daily in Pl. 2, fig. 2.

The group treated with testosterone shows a range of 9.8-16.2 in the figures for average cell count per alveolus, with a mean of 12.4. There is clearly a large increase in size of alveoli. Increased activity is also manifested by densely packed nuclei indicating active division of cells in the basement membrane region of the alveolus and by rupture and breakdown of cells in the mouth of the alveolus, as may be seen in Pl. 2, fig. 3.

This method of interpretation does not indicate whether changes have taken place in alveolar number, for which purpose the following analysis is put forward.

If  $n$  is the number of alveoli per unit area of skin, and  $d$  is the mean diameter, then the alveolar count will be proportional to  $nd$  on the average. If we assume that the sebaceous cell size does not alter much from group to group, as appears to be the case,

Table 1. *Sebaceous cell and alveolar counts of female albino rats treated with oestradiol benzoate and testosterone propionate. Figures given are for 15 mm. of section*

Rat no.	Treatment	Dose per day	Duration (days)	Sebaceous cell count	Alveolar count	Average cell count per alveolus		
S 49	Control	—	39	303	36	8.4		
S 50			39	302	32	9.4		
S 51			39	172	28	6.1		
S 52			39	254	28	9.1		
S 53			39	234	27	8.7		
S 54			39	210	34	6.2		
V 1			40	252	40	6.3		
V 2			40	288	38	7.6		
V 3			40	335	41	8.2		
V 4			40	426	67	6.4		
Mean				277.6	37.1	—		
s.d. of observations				67.5	11.0	—		
S 1	Oestradiol benzoate	1 µg.	36	230	29	7.9		
S 2			36	168	24	7.0		
S 3			36	182	23	7.9		
S 4			36	208	32	6.5		
S 6			36	94	20	4.7		
V 5			37	162	28	5.8		
V 6			37	208	32	6.5		
V 7			37	145	31	4.7		
V 8			37	208	34	6.1		
Mean				178.3	28.1	—		
s.d. of observations				39.5	4.6	—		
S 8	Oestradiol benzoate	100 µg.	36	74	19	3.9		
S 9			36	112	33	3.4		
S 10			36	119	26	4.6		
S 11			36	77	17	4.5		
S 12			36	149	29	5.0		
V 9			40	40	16	2.5		
V 11			40	85	30	2.8		
V 12			40	62	18	3.4		
V 13			40	81	27	3.0		
V 14			40	108	23	4.7		
Mean				90.7	23.8	—		
s.d. of observations				29.9	5.7	—		
S 13			Testosterono propionate	1 mg.	36	654	50	13.1
S 14					36	767	48	16.0
S 15	36	386			33	11.7		
S 16	36	699			53	13.2		
S 17	36	612			47	13.0		
S 18	36	615			38	16.2		
V 15	35	483			48	10.1		
V 16	35	455			39	11.7		
V 17	35	636			65	9.8		
V 18	35	414			41	10.1		
V 19	35	659			51	12.9		
Mean					580.0	46.6	—	
s.d. of observations					119.2	8.3	—	



we may, as a first hypothesis, suppose that alveolar size depends on the number of cells per alveolus. If this is so, then alveolar volume is proportional to  $N$ , where  $N$  = mean number of cells per alveolus. But  $d$  is proportional to cube root of volume and hence to  $\sqrt[3]{N}$ .

Let us now test the assumption that alveolar number ( $n$ ) is constant. In this case the alveolar count will be proportional to  $d$  only, and  $N$  will be proportional to the sebaceous cell count. Hence we should find that alveolar count is proportional to  $d$  which is proportional to  $\sqrt[3]{N}$  which is proportional to  $\sqrt[3]{(\text{Sebaceous cell count})}$ , i.e.

$$\frac{\text{Alveolar count}}{\sqrt[3]{(\text{Sebaceous cell count})}} = \text{constant.}$$

This may be tested with reference to the mean figures from Table 1, when we find:

Group	$\sqrt[3]{(\text{Sebaceous cell count})}$	Alveolar count	$y/x$
	$x$	$y$	
Control	6.5	37	5.7
Oestradiol benzoate (1 $\mu$ g.)	5.6	28	5.0
Oestradiol benzoate (100 $\mu$ g.)	4.5	24	5.3
Testosterone propionate (1 mg.)	8.3	47	5.7

It seems evident that the alveolar number is constant; in which case changes in either sebaceous cell count or alveolar count are to be interpreted as indicating changes in alveolar size only.

Table 2. *Sebaceous cell and alveolar counts of female albino rats treated with progesterone*

Rat no.	Treatment	Dose per day	Duration (days)	Sebaceous cell count	Alveolar count	Average cell count per alveolus
U 1	Control	—	20	237	32	7.4
U 2				414	46	9.0
U 3				306	36	8.5
U 4				199	33	6.0
U 5				276	38	7.3
U 6				355	48	7.4
U 7				236	38	6.2
U 8				380	49	7.8
U 9				151	32	4.7
U 10				231	31	7.4
		Mean		278.5	38.3	—
		S.D. of observations		79.8	8.9	—
U 11	Progesterone	0.1 mg.	20	197	32	6.1
U 12				316	41	7.7
U 13				339	43	7.9
U 14				279	35	8.0
U 15				331	44	7.5
		Mean		292.4	39.0	—
		S.D. of observations		52.0	4.7	—
U 16	Progesterone	0.5 mg.	20	327	49	6.7
U 17				203	32	6.3
U 18				223	37	6.0
U 19				298	39	7.6
U 20				275	44	6.2
		Mean		265.2	40.2	—
		S.D. of observations		45.1	5.8	—



FIG. 1. Sebaceous gland of normal female albino rat (S 51).  $\times 510$ .



FIG. 2. Sebaceous gland of female rat treated with 100  $\mu$ g. per day of oestradiol benzoate for 36 days (S 8).  $\times 510$ .

FIG. 3. Sebaceous gland of female rat treated with 1 mg. of testosterone propionate per day for 36 days (S 18).  $\times 510$ .

There is no evidence (Table 2) that progesterone significantly affects the glands.

The validity of the differences is not prejudiced by the fact that in any section not all of the glands will be cut medially.

#### DISCUSSION

These observations may be compared with those of Hooker & Pfeiffer [1943] who, in long-period experiments on hair growth during which rats were treated with 83  $\mu$ g. of oestradiol benzoate twice weekly, noted that atrophy of the sebaceous glands had occurred when the animals were killed after 6–10 months. It is clear that this effect was produced by massive long-period dosage, although the present work shows a limited reduction of activity brought about by doses of 1  $\mu$ g. daily for 36 days. This is of special interest in the light of observations by Bullough [1946] who, working on mice, found that the immediate effect after only two doses of 25  $\mu$ g. of oestrone was an increased number of mitoses in the sebaceous gland.

Hooker & Pfeiffer found that atrophy of sebaceous glands was not seen in animals treated with both oestrogen and testosterone, but they emphasize that their study throws no light on the action of androgen when administered alone.

Since the present work was completed, the papers of De Graaf [1942, 1943], referred to by Gaarenstroom & De Jongh [1946], have come to notice. De Graaf [1942] found that moderate development of the sebaceous glands could be induced by both oestrone and testosterone in the nipple of the guinea-pig, but that extensive growth occurred when male hormone combined with a small dose of oestrogenic substance was used. Working on rats [1943] he found that the sebaceous glands, particularly those of the back region, developed under the influence of testosterone.

It is felt that these actions of the sex hormones have some bearing on the etiology and treatment of acne and, further, on the degenerative skin changes of old age. It should be borne in mind that the sex hormones are probably not the only factors influencing the sebaceous glands, and work is at present in progress using thyroid and pituitary hormones.

A comparison of changes in the mammary glands might be of interest. In some of the animals in the present experiments mammary gland preparations were made. It was found that whereas 1  $\mu$ g. per day of oestradiol benzoate stimulated mammary duct growth, 100  $\mu$ g. caused stunted growth; both doses reduced sebaceous growth. Testosterone caused growth of both mammary ducts and lobules, and sebaceous glands.

#### SUMMARY

1. A technique for estimating the degree of development of sebaceous glands in the rat is described.
2. Oestradiol benzoate in massive doses (100  $\mu$ g. daily for 36 days) causes atrophy of the sebaceous glands in the female rat.
3. Oestradiol benzoate in moderate doses (1  $\mu$ g. daily for 36 days) has a less marked effect, but is shown to decrease the total amount of gland material.
4. Testosterone propionate (1 mg. daily) increases the size and activity, but not the number, of the glands.
5. There is no evidence that progesterone affects the glands.

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# THE ROLE OF THE ADRENAL GLANDS IN PROTEIN CATABOLISM FOLLOWING TRAUMA IN THE RAT

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During the war years extensive observations have been made on patients suffering from various forms of trauma. The changes in protein metabolism have received special interest since Cuthbertson [1930, 1936] emphasized the marked negative nitrogen balance that regularly occurs after severe trauma. The numerous clinical papers on this subject have recently been reviewed by Howard [1945].

Since the original experimental observations of Long, Katzin & Fry [1940] showing that the cortex of the adrenal gland was intimately concerned with the conversion of protein to carbohydrate, considerable evidence has been put forward to show that this mechanism may be involved in the metabolic changes taking place after various traumatic procedures. Albright [1943], in a consideration of Cushing's syndrome, discussed the possible elaboration of sugar-forming and protein-fixing hormones by the adrenal glands. An increased secretion of the former, resulting in gluconeogenesis from protein, might be responsible for the increased nitrogen excretion occurring after various types of damaging stimuli. This increased production of glucose was suggested as a protective type of reaction. Other workers have considered the role of the adrenal cortex in defence mechanisms. Thus the hypertrophy of the adrenal glands and evidence of hypersecretion observed in response to injury has formed the basis of the alarm reaction and adaptation syndrome extensively studied by Selye [1946] and his associates. The breakdown of lymphoid structures and the lysis of lymphocytes as a protective response has been shown by White & Dougherty [1946] to be related to the liberation of adrenal cortical hormones. Changes in the ascorbic-acid content of the adrenal glands have been found to occur rapidly after various forms of stimuli and have been regarded as indicating increased adrenal function [Sayers, Sayers, White & Long, 1943]. Finally a substance having a biological activity similar to the 11-oxygenated adrenal hormones has been recovered from the urine of patients who have undergone operative and other damaging procedures [Shipley, Dorfman & Horwitt, 1943; Venning, Hoffman & Browne, 1944].

Although the evidence that activity of the adrenal cortex and excretion of adrenal cortical hormones takes place in response to various damaging stimuli seems fairly convincing, there was no proof that the hormones thus secreted actually protected the organism or altered the protein metabolism. In fact, in a paper recently received, Ingle, Ward & Kuizenga [1947] report that although after leg fractures in the adrenalectomized rat an increase in nitrogen excretion does not take place, it does so if the animal is maintained on replacement therapy with cortical extract *at a basal level* (i.e. where there is no possibility of an increased supply of cortical hormones).

During studies on nitrogen metabolism in the rat after trauma it was noted that a large and consistent increase in nitrogen excretion followed an amount of trauma considerably less than that necessary to cause death [Noble & Toby, 1947*b*]. Although it was possible that the protein breakdown was caused by adrenal cortical hormones liberated in response to the trauma, this seemed unlikely since even large doses of an active adrenal cortical extract were followed by comparatively small increases in excreted nitrogen. It was decided therefore to attempt to ascertain what role the adrenal glands play in increasing the urinary nitrogen. In the initial experiments the nitrogen excretion of normal and adrenalectomized rats was compared before and after trauma, and the effect of treatment with adrenal cortical extract in similar groups of rats determined. In other experiments changes in urinary nitrogen excretion have been correlated with alterations in blood non-protein nitrogen (N.P.N.). The effects of two other substances, adrenaline and ascorbic acid, have also been studied.

#### METHODS

Hooded male rats raised in the laboratory and weighing 160–180 g. at the time of operation were used. They were fed a diet containing 15 % casein and 8 % yeast, found adequate for normal growth and previously described for use in nitrogen-excretion experiments [Noble & Toby, 1947*b*; Toby & Noble, 1947]. Except where indicated, all the adrenalectomized animals used were adequately maintained on 0.9 % saline; in a few cases glucose was added to make a 10 % solution. About a week after the experiment the rats were put on water, to check whether adrenal insufficiency was severe enough to cause death. About half of the rats of the above size in this colony survived adrenalectomy without receiving additional salt. The usual criteria of absence of pituitary function and inspection of the sella at autopsy were used to check the completeness of hypophysectomy.

Trauma was applied by two methods. Limb ischaemia was produced by clamping the hind legs as described by Haist & Hamilton [1944]. In a few experiments drum trauma was applied according to the method of Noble & Collip [1942], but, after exposure to each 100 turns in the drum a rest interval of 5 min. was interposed. Normal animals withstood a total of 1000 turns of such interrupted trauma without mortality [Toby & Noble, 1944]. Animals were treated with adrenal cortical extract—ACE (Connaught Laboratories—30 dog units per ml.), deoxycorticosterone acetate in oil—DCA, adrenaline (Parke Davis 1 : 1000), and ascorbic acid.

During the urine collections, which were begun immediately after the trauma, the animals were placed in individual Hopkins metabolism cages and received no food. The cages were placed on large funnels and the usual precautions taken to avoid faecal contamination or loss of nitrogen. The collection was divided into two periods of 10 and 14 hr. and was not prolonged beyond 24 hr. for fear of death in the operated animals. In some experiments with intact animals the collection was extended for another 10 hr. Determinations of total nitrogen and N.P.N. were done by the micro-Kjeldahl method. N.P.N. estimations were made on blood obtained by heart puncture. The volume of urine was recorded for each period. In the results reported, however, only the total excretion of nitrogen is given, expressed in relation to body weight at the beginning of the experiment. In most cases groups of 4 to 6 rats were run simultaneously and the results averaged. Although there was individual variation

in the control experiments with intact animals, the average values obtained from groups of 4 to 6 rats were almost identical for non-traumatized animals, and varied at most  $\pm 13$  mg. after trauma.

## RESULTS

*Effect of adrenal cortical extract on nitrogen excretion of normal and traumatized rats*

Cortical extract was administered to normal rats, and to rats that were traumatized either by the drum or the clamp method. In Table 1 it may be seen that ACE even in large doses caused an increase in nitrogen excretion of not more than 17 mg./100 g. in 24 hr. Although this change was consistently found, it was less than half that resulting from moderately severe trauma. ACE given to animals that had received drum trauma produced little or no alteration in the increase in nitrogen output. In a few experiments with clamped rats ACE treatment gave inconsistent results, but the number of animals used was too small for comparison.

Table 1. *Effect of adrenal cortical extract on nitrogen excretion of non-traumatized and traumatized intact rats*

No. of rats	Form of trauma	Treatment*	N excretion† (mg./100 g./24 hr.)
27	—	—	64
8	—	ACE 1 ml. subcutaneously hourly $\times$ 8 ( $\equiv$ 240 dog units)	81
6	Drum 1000 turns interrupted	—	107
6	Drum 1000 turns interrupted	ACE 1 ml. subcutaneously hourly $\times$ 8 ( $\equiv$ 240 dog units)	113
12	Clamps—3 hr.	—	111
3	Clamps—3 hr.	ACE 1 ml. subcutaneously hourly $\times$ 3	105
2	Clamps—3 hr.	ACE 1 ml. subcutaneously hourly $\times$ 3 repeated at 12 hr.	121

\* Begun at start of collection period.

† Ranges were in all cases within  $\pm 13$  mg./100 g./24 hr. of the mean.

*Nitrogen excretion of adrenalectomized rats*

A series of adrenalectomized animals was tested at intervals of from 15 min. to 31 days after operation and the results are shown in Table 2.

Table 2. *Nitrogen excretion of non-traumatized adrenalectomized rats*

No. of rats	Time after adrenalectomy	N excretion (mg./100 g./24 hr.)
3	15 min.	55 (49-58)
3	3 hr.	55 (49-59)
5	4 days	86 (69-92)
6	5 days	85 (67-94)
3	7 days	81 (72-93)
3	10 days	73 (60-87)
7	12-14 days	73 (64-83)
5	19 days	73 (67-81)
5	27 days	68 (60-79)
5	31 days	64 (48-85)



Immediately following removal of the adrenal glands there appeared to be a reduction in nitrogen excretion from the level of 64 mg./100 g. for control unoperated animals to 55 mg./100 g. After 4-5 days the excretion was somewhat elevated (86 mg./100 g.) and thereafter there was a gradual return to normal values over a period of 4 weeks. Although the average results of groups of rats showed this change, considerably more individual variation occurred in these animals than in intact ones, particularly at the time when the average output was highest.

*Nitrogen excretion of traumatized adrenalectomized rats*

Because of the susceptibility of the saline-treated adrenalectomized rat to trauma little more than half of such animals survived after 3 hr. clamping [Noble & Toby, 1947a]. In some of the experiments, therefore, the periods of clamping were shorter. The nitrogen excretion of adrenalectomized rats after trauma is shown in Table 3 and compared with that of intact controls and of animals in which only one adrenal gland was removed.

Table 3. *Nitrogen excretion of traumatized adrenalectomized rats*

No. of rats	Time after adrenalectomy	Duration of clamping (hr.)	Treatment	N excretion (mg./100 g./24 hr.)
22	(Intact controls)	3	—	108 (90-121)
11	(Intact controls)	3	Saline and glucose	100 (89-111)
4	(Intact controls)	1	Saline and glucose	67 (64-70)
3	One adrenal removed just before clamping	3	—	97 (89-110)
6	One adrenal removed day before clamping	3	—	103 (95-123)
2	15 min.	1	Saline and glucose	56 (54, 59)
3	15 min.	3	Saline	80 (78-84)
17	4-7 days	1½	Saline	77 (63-93)
2	5 days	3	Saline	90 (86, 94)
2	7 days	3	Saline	64 (58, 71)
3	7 days	3	Saline and glucose	59 (48-71)
3	22 days	3	Saline—concentration increased to 1.5% for 18 hr. preceding experiment	75 (74-75)

When the period of clamping was only 1 hr. there was little change in nitrogen excretion in intact rats (cf. Table 1), and in animals adrenalectomized 4 and 7 days before clamping of 1½ hr. duration the nitrogen excretion, although higher than in intact controls, was no higher than in unclamped adrenalectomized rats.

After 3 hr. clamping the average urinary nitrogen excretion differed little from that of untraumatized adrenalectomized rats (Table 2), but there was a greater individual variation in nitrogen excretion than in intact rats and an occasional animal showed an increased nitrogen output. The removal of only one adrenal gland did not alter the excretion of nitrogen after trauma.

Following the experiment, animals that survived were allowed a few days for recovery, and were then given water to drink instead of 0.9% NaCl. About half of these animals died, a proportion which is in keeping with that expected for this colony. Rats that survived, presumably because of accessory adrenal tissue, did not show any greater nitrogen excretion than did those that died of adrenal insufficiency.

*Effect of adrenal cortical extract on the nitrogen excretion of adrenalectomized and traumatized adrenalectomized rats*

In Table 4 the effect of treating adrenalectomized rats with ACE or DCA is shown and the results compared with those obtained when such rats were also traumatized.

Table 4. *Effect of adrenal cortical extract on nitrogen excretion of non-traumatized and traumatized adrenalectomized rats*

No. of rats	Time after adrenalectomy (days)	Duration of clamping (hr.)	Treatment	N excretion (mg./100 g./24 hr.)
4	4	—	DCA 1 mg./day	70 (64-73)
4	4	3	DCA 1 mg./day	72 (69-77)
2	7	3	DCA 1 mg./day for last 4 days	106 (103, 109)
5	7-11	3	Saline throughout and DCA 1 mg./day for last 4 days	79 (66-89)
5	14	3	Saline until last 4 days, DCA 1 mg./day for last 4 days	54 (47-62)
5	6	3	ACE 0.5 ml. t.i.d.	72 (66-76)
4	8	3	ACE 0.5 ml. b.i.d.	55 (41-62)
4	7	3	Saline and ACE 0.5 ml. t.i.d.	88 (84-96)
6	7	1½	Saline and ACE 0.5 ml. × 7 on day of exp.	82 (74-92)
2	8	—	Saline throughout. ACE 0.5 ml. twice on day before and q.i.h. × 6 on day of exp.	87 (82, 93)
4	8	3	Saline and ACE as above	90 (87-93)
5	10	—	Saline and ACE as above	71 (60-77)
4	10	3	Saline and ACE as above	106 (94-126)
7	11	—	Saline and ACE as above	78 (68-86)
5	11	3	Saline and ACE as above	102 (86-123)
5	12	3	Saline and ACE as above	94 (79-114)
7	19	—	Saline and ACE as above	67 (57-79)
5	27	3	Saline and ACE as above	81 (74-85)

DCA in oil and ACE were injected subcutaneously.

Treatment with DCA did not allow the normal response in nitrogen excretion to occur after trauma, except in one experiment where only two animals were used.

In a number of experiments in which ACE was used, rats showed an increased nitrogen excretion after trauma. The administration of 0.5 ml. three times daily as a maintenance dose, without saline, was apparently insufficient to allow the normal increase in urinary nitrogen after 3 hr. clamping, but nevertheless allowed the animals to withstand this amount of trauma. When rats were maintained on saline and the above dose of ACE daily the excretion following trauma was approximately the same as that of adrenalectomized control animals. However, when larger doses of ACE were given (0.5 ml. twice on the preceding day and six times during the first collection period) the nitrogen excretion rose, achieving approximately normal values in the traumatized groups used 10-12 days after operation. Control animals similarly treated but not clamped did not show any increase in nitrogen output.

*Changes in blood non-protein nitrogen and nitrogen excretion after trauma*

In a number of experiments the level of N.P.N. in the blood was determined to correlate this with nitrogen excretion. Table 5 shows the blood N.P.N. of intact rats at different intervals after clamping.

Table 5. *Nitrogen excretion and blood non-protein nitrogen after trauma*

No. of rats	Duration of clamping (hr.)	Treatment	N excretion (mg./100 g./24 hr.)	Blood N.P.N. (mg./100 ml.)		
				1-3 hr.	3-9 hr.	24 hr.
Intact rats						
3	—	None	—	—	—	52 (50-53)
3	3	None	—	53 (46-67)	—	—
4	3	None	—	—	56 (52-61)	—
2	3	None	92 (85, 98)	—	—	52 (46, 59)
Adrenalectomized rats*						
3	—	0.9% NaCl	74 (64-83)	—	—	51 (45-60)
7	1½	0.9% NaCl	72 (66-80)	—	—	39 (32-47)
6	1½	ACE 0.5 ml. × 7 during exp.	82 (74-92)	—	—	36 (34-38)
5	3	0.9% NaCl	—	95 (70-117)	—	—
6	3	0.9% NaCl	—	—	99 (82-108)	—
3	3	0.9% NaCl	92 (86-97)	—	—	33 (30-35)
4	3	ACE 0.5 ml. b.i.d.	55 (41-62)	—	—	111 (91-160)

\* 4-7 days after operation.

The N.P.N. did not change in intact rats in response to the trauma, although the nitrogen excretion was increased. Adrenalectomized rats (4-7 days after operation) were clamped for 1½ hr., some of the animals being treated with ACE. There was very little change in urinary nitrogen and the N.P.N. values were slightly below normal. When the clamps were applied for 3 hr., however, marked alterations were found. The N.P.N. rose in all animals that died within the 3 hr. and up to 9 hr. after release of the clamps, even though small amounts of urinary nitrogen were excreted. Three animals that survived the experiment excreted increased amounts of nitrogen in response to trauma, and did not show a high N.P.N. A group of animals maintained on small doses of ACE did not excrete excess nitrogen after trauma, as was found in some of the earlier groups of animals similarly treated and listed in Table 4. However, all these rats showed a large rise in N.P.N. Apparently, protein breakdown occurred in these animals and was manifest by high levels of N.P.N. in those that died soon after trauma. In some untreated animals excretion did occur. Even after a maintenance dose of ACE that enabled the animals to withstand trauma, urinary nitrogen was not increased, but the blood N.P.N. was high. Larger doses of ACE, as noted previously, allowed the excess nitrogen to be excreted.

*Effect of adrenaline and ascorbic acid on nitrogen excretion*

In view of the relation of adrenaline and ascorbic acid to the function of the adrenal gland some experiments were conducted to see whether these substances might affect nitrogen excretion. The results are listed in Table 6.

Table 6. *Effect of adrenaline and ascorbic acid on nitrogen excretion of intact rats*

No. of rats	Duration of clamping (hr.)	Treatment*	N excretion (mg./100 g.)	
			24 hr.	34 hr.
12	—	None	64	94
8	—	Adrenaline 1 : 1000, 0.02 ml./kg. subcutaneously × 4	87	—
2	—	Adrenaline 1 : 1000, 0.125 ml. i.p.	95	—
1	—	Adrenaline 1 : 1000, 0.250 ml./i.p.	105	—
1	—	Adrenaline 1 : 1000, 0.50 ml./i.p.	111	—
3	—	Ascorbic acid 250 mg. by stomach tube	51	63
4	—	Ascorbic acid 50 mg. subcutaneously q.i.h. × 3	58	72
3	—	Ascorbic acid 50 mg. subcutaneously q.i.h. × 3. ACE q.i.h. × 5	77	88
8	3	None	108	175
3	3	Ascorbic acid 50 mg. subcutaneously q.i.h. × 3	105	—
4	3	Ascorbic acid 250 mg. by stomach tube on day preceding experiment and again after removing clamps	93	122

\* Begun at start of experiment except where specified.

Adrenaline given to normal rats increased nitrogen excretion to an extent proportional to the dose injected. With the largest dose used the increased loss of nitrogen was as great as that occurring after the forms of trauma previously described (Table 1).

Ascorbic acid administered to normal rats lowered nitrogen excretion. This effect was especially marked when the urine collection was extended a further 10 hr. making the total period 34 hr. Over this time untreated, control rats excreted 94 mg./100 g. compared with 63 and 72 mg./100 g. in treated rats. When ascorbic acid was given in addition to ACE some reduction in the expected nitrogen excretion took place. When ascorbic acid was given to animals clamped 3 hr. there was a reduction in nitrogen excretion, especially over a 34 hr. period, the value of 122 mg./100 g. for 34 hr. being some 53 mg./100 g. less than the value obtained for untreated, clamped rats.

#### *Nitrogen excretion of hypophysectomized rats*

Because of the trophic control of the adrenal cortex by the pituitary gland it was thought of interest to examine the nitrogen excretion of hypophysectomized rats.

Table 7. *Nitrogen excretion of hypophysectomized rats*

No. of rats	Time after operation	Duration of clamping (hr.)	Treatment*	N excretion (mg./100 g./24 hr.)
2	Immediately	—	—	78 (69, 87)
2	3 hr.	—	—	65 (63, 66)
3	3 hr.	3	—	63 (57-66)
3	1½ hr.	1½	ACE 0.5 ml. subcutaneously hourly × 6	84 (79-89)
3	3 hr.	—	ACE 0.5 ml. subcutaneously hourly × 6	79 (76-82)
3	3 hr.	3	ACE 0.5 ml. subcutaneously hourly × 6	69 (54-97)
2	4 days	—	—	108 (102, 115)
4	6 days	—	—	108 (100-125)
2	2 weeks	—	—	89 (87, 90)
2	2 weeks	1½	—	59 (56, 61)
3	4 weeks	—	—	76 (72-82)
4	6 weeks	—	—	75 (72-92)

\* Begun at start of experimental period.

Four to six days after removal of the pituitary gland the nitrogen excretion rose (Table 7). This was about the same time after operation as the highest output seen in adrenalectomized animals. Some increase was still present after 2 weeks, but the values approached normal after 4 weeks. The increased excretion did not appear to be related to the weight loss after hypophysectomy, which was not unduly large. Some hypophysectomized animals were clamped, but no increase in nitrogen excretion was observed (Table 7). Treatment with ACE after clamping did not restore the normal nitrogen response.

#### DISCUSSION

The experiments that have been described were designed to determine what part the adrenal cortex might play in the increased protein breakdown occurring after trauma. Initially the urinary output of nitrogen was considered an indication of protein catabolism in the animal. However, because of the inability of the adrenalectomized animal to excrete the excess nitrogen, it later became necessary to estimate the changes occurring in the N.P.N. of the blood as well as in the urinary nitrogen.

A number of workers have studied nitrogen excretion in adrenalectomized rats. Rubin & Krick [1936] found such animals to show a negative nitrogen balance, which could be reduced by adequate NaCl therapy. Sandberg & Perla [1936], using adrenalectomized rats maintained in good health by dietary measures, noted an increased nitrogen excretion and lowered retention of ingested nitrogen for prolonged periods after operation. Evans [1936], Harrison & Long [1939] and Long, Katzin & Fry [1940] all reported a decreased nitrogen excretion in adrenalectomized rats. The latter workers and Sprague [1940] also found that the injection of ACE was followed in both normal and adrenalectomized rats by an increased excretion of nitrogen in the urine.

In the results reported in this paper, there seems to be evidence that for a brief period after adrenalectomy nitrogen excretion may be below normal, but that for some weeks thereafter higher values than normal are obtained. After about 4 weeks, the normal level is re-established. The results obtained on injection of ACE agree with those of other workers in that an increased excretion of nitrogen takes place.

Relatively little experimental work, however, has been done on the nitrogen excretion of adrenalectomized animals after exposure to trauma. In agreement with the observations of others, it was found that after two different types of trauma in the intact rat, a consistent elevation in urinary nitrogen occurred [Noble & Toby, 1947*b*]. The amount of nitrogen excreted after only moderate amounts of trauma was about double that produced by relatively large doses of ACE in normal animals. Also when ACE was given to traumatized animals, it was not possible to increase the urinary nitrogen output. These observations did not appear to support the suggestions, previously discussed, that under the stimulus initiated by trauma, a secretion of the adrenal cortex is induced which causes increased protein breakdown and a resulting loss of nitrogen in the urine. Experiments in which treated and untreated adrenalectomized animals were subjected to trauma were necessary to determine the role of the adrenal in protein catabolism. Difficulty, however, was experienced because of the marked susceptibility to trauma of the adrenalectomized rat, even when maintained in good health by salt.

Apparently clamping the hind legs for 1½ hr., a procedure which most adrenalectomized rats could withstand, was not sufficient to cause an increased nitrogen excre-

tion, or to elevate the blood N.P.N. A number of animals did survive 3 hr. clamping, and in this case, although the average nitrogen excretion was not increased, it was noted that in some animals an increase did take place. The explanation of this inconsistency was apparent in later experiments in which estimations of blood N.P.N. were also made. In this case the N.P.N. values were consistently increased in animals that did not excrete excess nitrogen in the urine. In instances where high urinary nitrogen values were obtained, the blood N.P.N. was normal. These results therefore indicate that after trauma the adrenalectomized as well as the intact rat shows a breakdown of protein. In most cases, however, there is retention of nitrogen and only in animals with good kidney clearance does a high excretion of nitrogen take place. Animals that do not excrete the excess nitrogen nevertheless, after the usual preliminary few hours of anuria, excrete a normal volume of urine. Although adrenalectomized rats treated with ACE tolerate as much trauma as intact animals, with the daily maintenance dose used in the experiments it was found that after trauma a marked rise in blood N.P.N. took place, but that the extra nitrogen was not cleared by the kidney. When larger doses of ACE were given after trauma, an increased excretion of nitrogen of the same magnitude as that occurring in intact animals resulted. These experiments therefore indicate that the adrenal cortex is not essential for the occurrence of protein catabolism after trauma, but that adequate amounts of adrenal cortical extract are, however, necessary in order that the kidney may excrete the nitrogenous end-products.

There seems no evidence from these results that the adrenal cortex is stimulated by trauma in order that its hormones may be used in the protein catabolic process. In a recent paper, Ingle *et al.* [1947] have described balance experiments on adrenalectomized rats, using a forced-feeding technique to ensure a constant intake. They noted that after leg fractures an increased urinary nitrogen excretion did not take place. An increased breakdown of protein was not, however, ruled out, since blood N.P.N. estimations were not done. After suitable treatment with cortical extract a normal nitrogen excretion was obtained in response to trauma. They concluded that the negative nitrogen balance was not caused specifically by an increased secretion of the cortical hormones.

Adrenaline was found to cause an increase in urinary nitrogen. Large doses approaching toxic levels were required to bring about an increase similar to that obtained after the amount of trauma used in our experiments. Ascorbic acid, on the other hand, appeared to depress nitrogen excretion and to diminish markedly the excess nitrogen loss resulting from trauma. Curiously enough, this substance was more effective than various proteins and amino-acids, which might be expected to cause a reduction in nitrogen excretion after trauma [Noble & Toby, 1947c].

Hypophysectomy, in the few experiments presented, produced more exaggerated changes in nitrogen excretion than did adrenalectomy, but the peak of excretion in both types occurred at the same time after operation. Treatment with ACE in amounts effective for replacement therapy in adrenalectomized rats was not adequate to restore the normal excretion of urinary nitrogen after trauma in the hypophysectomized animal.

The evidence which this paper presents of a physiological role of the adrenal gland in the protein catabolic response occurring after trauma is essentially negative. One

cannot but wonder what function the increased excretion of the adrenal cortex serves after trauma, since the evidence is so suggestive that the organ is stimulated. It seems certain that an increased secretion of adrenal hormones does not improve the tolerance of a normal animal to traumatic procedures, nor is it necessary for the breakdown of protein. It is possible that its function is more specifically related to the breaking down of lymphocytes and release of antibodies [White & Dougherty, 1946] or to its action in aiding the animal in the development of resistance to trauma [Noble, 1943].

#### SUMMARY

The urinary excretion of rats given large doses of adrenal cortical extract (240 dog units) did not approach the high level reached after moderately severe trauma. Treatment with adrenal cortical extract did not increase the nitrogen output of traumatized animals.

In the adrenalectomized rat the daily nitrogen output dropped slightly after operation and then rose above normal levels, reaching a peak on the 4th or 5th day and returned slowly to normal over a period of 4 weeks.

The urinary nitrogen response to trauma was unaffected when one adrenal only was removed. Bilaterally adrenalectomized animals showed on the average no elevation of urinary nitrogen after trauma, although a response occurred in a few individual cases. Blood N.P.N. values, however, were consistently increased in those not showing increased nitrogen output.

Adrenalectomized animals treated with maintenance doses of cortical extract showed no excess urinary nitrogen after trauma, but the N.P.N. of the blood was increased. With larger doses of cortical extract an elevation of urinary nitrogen after trauma was found, comparable with that found in intact rats. The amount of cortical extract used did not affect the nitrogen excretion of untraumatized adrenalectomized animals. Protein catabolism after trauma was apparently not dependent on the adrenal cortex.

Adrenaline in large doses increased the excretion of nitrogen, the response being roughly proportional to the dose.

Ascorbic acid depressed the nitrogen output of normal and traumatized rats.

A small group of hypophysectomized animals showed the same type of elevation as was found in adrenalectomized rats. No increase in urinary nitrogen occurred after trauma, even when adrenal cortical extract was administered.

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# FURTHER NOTES ON THE ACTION OF OESTRONE AND RELAXIN ON THE PELVIS OF THE SPAYED MOUSE, INCLUDING A SINGLE-DOSE TEST OF POTENCY OF RELAXIN

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Our earlier work on the effect of relaxin upon the symphysis pubis of the mouse was planned with the object of reproducing in the spayed female, during a similar time interval, the changes which take place naturally during the last 6 days of pregnancy and which were described in a previous paper [Hall & Newton, 1946]. We have shown [Hall & Newton, 1947] that daily injections of 0.2 ml. of relaxin extract given for 5 days simultaneously with daily administration of oestrone, after 2 days' priming with oestrone alone, will produce an interpubic separation in spayed animals of the same order as that which occurs at the end of normal pregnancy.

During subsequent work it has often been necessary to determine the potency of individual batches of relaxin extract, and it was felt that a more convenient method of assay, involving the use of smaller quantities of extract, was needed. The discovery that a single injection of relaxin extract, following several days' priming with oestrone, would produce a measurable and reasonably constant symphysial response, led to a more detailed investigation into the possibility of a convenient single-dose assay in the mouse, on the lines of that used by Abramowitz, Money, Zarrow, Talmage, Kleinholz & Hisaw [1944] in the guinea-pig.

Experiments were planned with the aim of finding the optimum duration of priming with oestrone after which maximal response to a single injection of relaxin would occur. This work has also yielded further information, included in this paper, concerning the action of oestrone on the symphysis.

## METHODS

The mice were albinos of the Parkes strain and were all virgin females. The ovaries were removed after sexual maturity.

Oestrone (B.D.H.) was weighed out, taken up in acetone, dissolved in ground-nut oil and the acetone removed *in vacuo*. The stock solution was diluted with ground-nut oil so that the volume injected was 0.05 ml. The relaxin extract was prepared from the serum of pregnant rabbits by the method of Abramowitz, Hisaw, Kleinholz, Money, Talmage & Zarrow [1942]. Details have been given in a previous paper [Hall & Newton, 1947]. The rabbits were usually bled twice, on the 27th or 28th and 29th or 30th days of pregnancy. The final precipitate containing the relaxing substance was dissolved in a quantity of water sufficient to make a volume of solution equivalent to one-fifth the volume of serum used. This is a slight variation from our original method of adding the solute on a weight-equivalent basis, and results in a similar but more constant concentration.

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The experiments were arranged so that a single injection of 0.2 ml. of relaxin extract was given approximately 24 hr. after the last of from two to sixteen daily injections of 1.5  $\mu$ g. of oestrone. When the number of days' treatment with oestrone varied within a single experiment, the dates of the first injections of oestrone were arranged so that the dose of relaxin was given on the same day to all the mice. All injections were given subcutaneously. X-ray examination of the pelvis was made before the start of the experiment, and immediately before and 24 hr. after the administration of the single dose of relaxin. The method of measuring the interpubic gap from X-ray photographs has been described in a previous paper [Hall & Newton, 1946]; it provides an exact and quantitative means of measuring the changes which occur throughout the course of any experiment.

## RESULTS AND CONCLUSIONS

*Effect of a single injection of 0.2 ml. of relaxin extract given 24 hr. after varying numbers of daily injections of 1.5  $\mu$ g. of oestrone in previously untreated, virgin, spayed mice*

The data given in Table 1 are compiled from a number of experiments in which seven different batches of relaxin extract were used. The numbers given in col. iii refer to the reference number of the batch of relaxin extract. The two main experiments

Table 1. *Effect on the symphysis pubis of previously untreated, virgin, spayed mice of a single injection of 0.2 ml. of relaxin extract given 24 hr. after varying numbers of daily injections of 1.5  $\mu$ g. of oestrone*

(i) No. of group and (in brackets) no. of exp.	(ii) No. of daily in- jections of oestrone	(iii) Serial no. of relaxin extract used	(iv) No. of mice used	(v) Average width of interpubic gap im- mediately before administra- tion of relaxin (mm.)	(vi) Average width of interpubic gap 24 hr. after administra- tion of relaxin (mm.)	(vii) (viii) Average increase in width of gap during last 24 hr. (mm.)	
						In each experiment	In each group
I	2	26	5	0.21	0.52	0.31	0.31
II (i)	4	26	4	0.26	0.95	0.69	0.59
(ii)		43	7	0.19	0.73	0.54	
III (i)	6	26	5	0.47	1.26	0.79	0.73
(ii)		43	10	0.29	0.99	0.70	
IV	7	27	7	0.23	1.37	1.14	1.14
V (i)	8	26	5	0.41	1.88	1.47	1.40
(ii)		39	5	0.10	1.50	1.40	
(iii)		43	10	0.39	1.75	1.36	
VI (i)	9	24	5	0.34	1.86	1.52	1.36
(ii)		28	6	0.44	2.47	2.03	
(iii)		30	10	0.23	1.11	0.88	

were those in which extracts no. 26 and 43 were used, and included respectively four and three groups of mice which received two, four, six and eight daily injections of oestrone before the administrations of relaxin. Table 1 also includes the results of five other experiments in which relaxin was given after 7, 8, or 9 days' treatment with oestrone. The number of mice used in each group of each experiment is shown in

col. iv. In cols. v and vi are given the average measurements in mm. of the interpubic gap immediately before and 24 hr. after the administration of relaxin, and in col. vii the average increase in the width of the gap during this last 24 hr. Col. viii gives the average increase during the final 24 hr. for all mice in each group.

The question of the time following the injections of relaxin at which the end-point should be read deserves mention. Abramowitz *et al.* [1944] found that in the guinea-pig the action of relaxin is maximal at 6 hr., and that the symphysis returns to its original condition in 24 hr. They therefore determine the response 6 hr. after the injection of the test material. X-ray measurements of the nineteen mice in Table 1 receiving relaxin 26 were made 6 and 24 hr. after the relaxin was given. The increase after 6 hr. was infinitesimal. We have also shown [Hall & Newton, 1947] that 48 hr. after stoppage of injections the gap has already started to close. It therefore appears that in mice the response should be measured after 24 hr.

The results show:

(i) In previously untreated, spayed, virgin mice, oestrone alone in daily doses of  $1.5 \mu\text{g.}$ , administered for periods up to 9 days, produces a slight interpubic separation of not more than 0.5 mm. This agrees with our earlier results [Hall & Newton, 1947].

(ii) Twenty-four hours after the injection of a single dose of 0.2 ml. of relaxin extract into these mice the interpubic gap has widened in all groups. That this final separation is produced by the relaxin, and is not merely a continuation of a process started by oestrone, is shown by the figures in col. v; e.g. eight daily injections of oestrone produce no greater effect than do six.

(iii) The effect of a single injection of relaxin increases progressively as the period of oestrone priming is prolonged. The different batches of relaxin extract used gave results in close agreement in each group, with the exception of group VI. The reason for the comparatively low potency of relaxin 30 is obscure, but, as will be shown later, there is some reason to believe that prolonging the duration of priming with oestrone beyond 8 days does not produce an increased response by the symphysis to relaxin.

*Effect of oestrone alone in spayed mice in which an interpubic separation had been experimentally produced at some previous date*

Table 2 shows the effect of eight and sixteen daily injections of  $1.5 \mu\text{g.}$  of oestrone alone on the symphysis pubis of spayed mice which had been treated with various combinations of oestrone and relaxin at some previous date and subsequently rested for 1 month (nos. 6, 19, 9, 16) or 2 months (all the others). The wide variation in the width of the interpubic gap in col. iii is accounted for by the fact that the mice were from several different experiments and had received varied treatment. In the interval between the end of the previous experiment and the start of the present one this gap had completely closed in all except four mice of group III. (A measurement of a quarter of a mm. is classed as 'closed'. It is often found in virgin, untreated females and is apparently due to the presence of articular hyaline cartilage.)

In groups I and II, Table 2, the administration of  $1.5 \mu\text{g.}$  of oestrone daily for 8 days produced in more than half the mice an interpubic separation of more than 0.5 mm. (0.8–2.0 mm.). In seven of these mice (group II) the injections were continued for a further 8 days, producing in three of the mice still further separation. In both groups a positive response was obtained only when an interpubic separation had been produced in the earlier experiment, while none of the symphyses which had not opened previously responded to oestrone. In our past and present experiments we

have not found that treatment with oestrone alone for this length of time will evoke in virgin, spayed mice an interpubic separation of more than 0.5–0.8 mm. It appears, therefore, that when the pubic bones have once separated, even though the gap may have since closed, a considerable reaction can be produced by oestrone alone. This conclusion is strengthened by the results of treatment with oestrone of four mice in group III (Table 2). At the close of the previous experiment an interpubic separation of 3.0–4.0 mm. had been produced (col. iii), and in three of the mice this gap had subsequently only partially closed (col. iv). After treatment with oestrone the average width of the gap increased from 1.08 to 2.05 mm. at the end of 8 days and to 3.28 mm. at the end of 16 days. In a fifth mouse (not included in the table as its previous history was unknown) the gap increased from 3.8 to 5.0 mm. after seven daily injections of oestrone.

Table 2. *Effect of daily administration of 1.5 µg. of oestrone alone on the symphysis pubis of spayed mice in which an interpubic separation had been experimentally produced at some previous date*

(i) No. of group	(ii) Serial no. of mice	(iii) Width of interpubic gap at close of previous experiment	(iv) (v) (vi) Width of interpubic gap in present experiment		
			Immediately before first injection of oestrone	24 hr. after last of 8 oestrone injections	24 hr. after last of 16 oestrone injections
I	6	2.0	0.25	2.0	—
	313	0.3	0	0.5	—
	331	1.3	0	0.5	—
	338	0.3	0	0.25	—
	348	2.5	0.25	0.8	—
	351	0.6	0	0.5	—
II	19	2.0	0.3	0.8	1.3
	314	0.9	0	0.3	0.3
	334	1.8	0	1.5	2.8
	337	1.2	0.25	0.8	0.8
	345	2.2	0.3	1.0	0.8
	347	0.3	0	0.3	0.3
	349	1.5	0	0.8	2.0
III	9	4.0	1.0	2.8	3.3
	16	3.0	0.3	1.0	2.3
	330	4.0	1.0	2.4	3.5
	344	3.5	1.0	3.0	4.0

*Effect of a single injection of relaxin after varying numbers of injections of oestrone in spayed mice in which an interpubic separation had been experimentally produced at some previous date*

All the mice included in Table 2 were given a single injection of 0.2 ml. of relaxin extract 24 hr. after the last injection of oestrone. The results are summarized in Table 3, which includes also a group of five mice which received only 2 days' priming with oestrone. The same relaxin extract was used throughout this experiment. The figures in col. vii show the average increase in the width of the interpubic gap 24 hr. after the administration of relaxin. The figures for groups I and II are in agreement with the average values for corresponding groups in Table 1, and it appears therefore that a positive reaction to the priming doses of oestrone does not affect the reaction to a subsequent single injection of relaxin.

Table 3. *Effect on the symphysis pubis of a single injection of 0.2 ml. of relaxin extract, given 24 hr. after varying numbers of daily injections of 1.5  $\mu$ g. of oestrone, in spayed mice in which an interpubic separation had been experimentally produced at some previous date*

(i) No. of group	(ii) No. of daily injections of oestrone	(iii) No. of mice used	(iv)	(v)	(vi)	(vii)
			Average width of interpubic gap (mm.)			
			At end of previous experiment	Immediately before injection of relaxin	24 hr. after injection of relaxin	Increase during last 24 hr.
I	2	5	1.36	0.37	0.72	0.35
II	8	6	1.17	0.76	2.37	1.61
III	16	7	1.41	1.19	2.54	1.35
IV	16	4	3.63	3.28	5.28	2.00

The data in Table 3 also appear to support the suggestion that (with the doses used) 8 days probably represents the optimum period of preliminary priming with oestrone after which maximal response to a single injection of relaxin will occur. In group III, continuation of the preliminary oestrone treatment for 16 days was not followed by a greater, but even by a slightly smaller response to subsequent administration of relaxin. It is true that in group IV the average increase (col. vii) is slightly higher than in group II, but these four mice are perhaps not strictly comparable with those of other groups, as an interpubic gap was still present at the start of the experiment.

#### DISCUSSION

During the course of a large number of experiments on the action of relaxin on the symphysis pubis of the mouse which have been carried out in this laboratory, the importance of using virgin mice in which separation of the pubic bones has never taken place has become increasingly apparent. In earlier papers [Hall & Newton, 1946; Hall, 1947] we have stated that after parturition the symphysis never completely returns to the virgin condition, but a small permanent separation remains. It is now evident that some time after an experimentally produced interpubic separation the gap may completely close. A closed symphysis on an X-ray photograph is therefore not a certain indication that the pelvis has never reacted to some relaxing agent in the past. It seems probable that complete re-closure does not normally occur after full separation, but that the symphysis may return to the virgin condition\* if the separation has been of only moderate degree.

Furthermore, after the pelvis has once reacted to the influence of relaxin, it becomes subsequently more sensitive to the action of oestrone and separation may proceed much faster than is normally the case with oestrogen alone.

Abramowitz *et al.* [1944] have described a modification of their method of assay of relaxin in the guinea-pig, in which they maintained the animals under 'constant oestrogen facilitation' and tested relaxin every third day. This method was abandoned because the animals became increasingly sensitive to the relaxin dosage and because

\* Histological investigation of these symphyses has not yet been carried out. It may be that although the gap disappears, the histological picture is not completely reversible.

an increased number of 'spontaneous' relaxations occurred before the relaxin was administered. It seems possible that, in fact, the animals having once responded to relaxin were becoming increasingly sensitive not to that hormone but to the oestrogen, and that this might account also for the 'spontaneous' relaxations.

Talmage [1947] states that in the response of the guinea-pig symphysis pubis to combinations of oestrogen and relaxin, two distinct types of changes are produced. He maintains that oestrogen produces a proliferative, relaxin a 'breakdown' reaction. In the spayed mouse [Hall, 1947] treatment for 1 week with relaxin and oestrone combined produced histological changes identical with those which occur at the end of normal pregnancy, whereas oestrone alone given for the same length of time left the histological picture unchanged. Interpubic separation produced by oestrogens alone has been described by several authors in the guinea-pig and by Gardner [1936] in the mouse, but, as Hisaw, Zarrow, Money, Talmage & Abramowitz [1944] have pointed out, large doses and prolonged treatment have been required.

The increased susceptibility to oestrone following a previous opening cannot be due to the opening itself having broken down some obstacle in a gross mechanical sense, for if it were so, opening under the action of oestrone alone (in the previously untreated, virgin animal) would proceed much faster than it actually does once a slight separation had occurred. On the contrary, enhanced sensitivity to oestrone may be found in the absence of any visible separation; some permanent change due to the previous opening process therefore persists in a concealed form. It is unwise to speculate about the nature of the change until susceptible symphyses have been examined histologically, but it is probably dependent on relaxin and may not be histologically identifiable. The slight opening which occurs in spayed animals treated with relaxin alone may well depend on small extra-ovarian supplies of oestrogen. The picture as a whole is consistent with the possibility that relaxin facilitates the action of oestrone, perhaps by removing some barrier to its action, and that this facilitation is in part permanent. We have already shown that our relaxin extracts are able to augment at least one reaction to oestrone [Dewar, Hall & Newton, 1946], and subsequent work as yet unpublished has confirmed this.

When administered alone to spayed mice for periods up to 2 weeks, relaxin produced separation of not more than 0.6 mm. [Hall & Newton, 1947, and later unpublished experiments]. The present experiments suggest that for maximal response a certain level of concentration of oestrogen is required. According to Kreitmair & Sieckmann [1939] in spayed rats and mice the oestrogenic effect of a single dose of 13  $\mu$ g. of oestrone lasts for 3-4 days only; Emmens [1939] states that 200  $\mu$ g. or more is needed to give an action lasting for a week. Considerable data collected in this laboratory indicate that the effect of a single injection of up to 2  $\mu$ g. of oestrone, as tested on the vaginal smear, is maximal early on the third day and then falls rapidly. On the basis of these results, it seems possible that when 1.5  $\mu$ g. of oestrone is administered daily for 8 days, some summation of effect may occur, but that, as the result of rapid excretion, augmentation is short lived and a constant level of response soon attained. If this were the case, then any further prolongation of the priming period beyond a certain duration would not enhance the final response.

The single-injection technique described should prove a convenient method of assaying the potency of relaxin extracts in the mouse. Although 8 days'

pre-treatment with oestrone appears to be the optimum period for maximal response to 0.2 ml. of relaxin extract, 5 days would probably be long enough for reliable results and would provide a more convenient method of assay.

#### SUMMARY

1. In spayed mice, a single injection of 0.2 ml. of relaxin extract given 24 hr. after the last of varying numbers of daily injections of  $1.5\mu\text{g}$ . of oestrone produced a measurable interpubic separation.

2. The effect of a single injection of relaxin increases progressively as the period of oestrogen priming is prolonged.

3. The optimum duration of preliminary priming with oestrone necessary for maximal response to this dose of relaxin is probably 8 days. It is suggested, however, that 5 days' pre-treatment might be sufficient for a reliable method of assay.

4. A closed symphysis on an X-ray photograph is not a certain indication that the pelvis has never reacted to a relaxing agent.

5. Evidence is given that when the pelvis has once reacted to relaxin, even though the gap may have since closed, it becomes subsequently more sensitive to the action of oestrone. In such animals separation may proceed much faster than is normally the case with oestrogen alone, though not so fast as during pregnancy or as when accompanied by relaxin, a rate of 2 mm. in 8 days being the highest so far recorded.

6. A positive reaction to the priming doses of oestrone does not alter the reaction to a subsequent single injection of relaxin.

7. A possible explanation of this acquired sensitivity of the symphysis to oestrone is discussed.

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#### STATISTICAL ANALYSIS OF THE DATA SUMMARIZED IN TABLES 1 AND 3

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The data are summarized in Table 4. (1) is the sum of squares of all observations in a group, (2) the sum and (3) the mean; (4), sum of squares from mean, is  $(1) - (2) \times (3)$ ; and (5) is the number in the group so that  $(3) = (2)/(5)$ . When a group is subdivided into two or three

smaller groups, say three, then (1)=(1A)+(1B)+(1C), (2)=(2A)+(2B)+(2C) and (5)=(5A)+(5B)+(5C), where A, B and C refer to the subdivisions, while (3) and (4) are calculated from the formulae given.

The subdivisions of a group appeared to be homogeneous except in group VI of Table 1. The sum of squares between A, B and C is calculated as (2A) (3A)+(2B) (3B)+(2C) (3C) - (2) (3); and is compared with (4A)+(4B)+(4C). Thus we find:

Source of variation	Sums of squares	Degrees of freedom	Mean square
Between A, B, C	5.08545	2	2.5427
Within A, B, C	8.6277	18	0.4793

The ratio of mean squares is 5.31, and if  $P$  represents the probability of a ratio as great or greater occurring when there is in fact no real difference between A, B, C,  $P$  lies between 5 and 1%. In other words, the difference between A, B, C is greater than chance will allow, i.e. group VI is not homogeneous. Consequently in testing the variation between groups of Table 1 we have omitted group VI.

As regards groups I-V of Table 1, the same method gave:

Source of variation	Sums of squares	Degrees of freedom	Mean square
Between groups I-V	8.438085	4	2.1095
Within groups I-V	20.0361	53	0.3780

The ratio of mean squares is 5.58 and corresponding  $P$  about 0.1%.

Evidence for difference between groups is thus fairly conclusive.

With the groups of Table 3 we find:

Source of variation	Sums of squares	Degrees of freedom	Mean square
Between groups	7.060965	3	2.3537
Within groups	6.3591	18	0.3533

The mean-square ratio is 6.66 and corresponding  $P$  less than 1%. Here, again, there appears no doubt about the difference between the four groups.

Table 4. *Statistical analysis of the data summarized in Tables 1 and 3*

No. of group and (in brackets) of experiment	4				
	1 Sums of squares	2 Sum	3 Mean	Sums of squares of deviations from mean	5 Number
From Table 1					
I	0.8475	1.5500	0.3100	0.3670	5
II (i)	2.5275	2.7500	0.6875	0.6369	4
(ii)	3.6725	3.7500	0.5357	1.6636	7
II	6.2000	6.5000	0.5909	2.3591	11
III (i)	4.0525	3.9500	0.7900	0.9320	5
(ii)	7.1900	7.0000	0.7000	2.2900	10
III	11.2425	10.9500	0.7300	3.2490	15
IV	12.8125	7.9500	1.1357	3.7836	7
V (i)	16.9225	7.3500	1.4700	6.1180	5
(ii)	11.7050	7.0000	1.4000	1.9050	5
(iii)	20.7100	13.6000	1.3600	2.2140	10
V	49.3375	27.9500	1.3975	10.2774	20
VI (i)	14.0050	7.6000	1.5200	2.4530	5
(ii)	27.0425	12.1500	2.0250	2.4387	6
(iii)	11.4800	8.8000	0.8800	3.7360	10
VI	52.5275	28.5500	1.3595	13.7131	21
From Table 3					
I	0.6825	1.7500	0.3500	0.0700	5
II	16.3725	9.6500	1.6083	0.8520	6
III	17.3300	9.5000	1.3571	4.4371	7
IV	17.0000	8.0000	2.0000	1.0000	4





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## PROCEEDINGS OF THE SOCIETY FOR ENDOCRINOLOGY

On 13 March 1948 the Third Annual General Meeting and Ninth Ordinary Meeting of the Society for Endocrinology took place at Guy's Hospital Medical School, London, S.E. 1.

The following Officers and Committee were elected for the Session 1948-9:

*Chairman of the Society*

Dr A. S. PARKES

*Hon. Secretaries*

Dr S. J. FOLLEY

Dr C. H. GRAY

*Hon. Treasurer*

Dr F. L. WARREN

*Hon. Editor of the Society's Proceedings*

Prof. S. ZUCKERMAN

*Members of the Committee*

Dr P. M. F. BISHOP

Dr P. L. KROHN

Dr I. W. ROWLANDS

Prof. F. G. YOUNG

In order to facilitate the transfer of control of the *Journal of Endocrinology* to the Society, certain changes in the Ordinary Rules of the Society were proposed and adopted.

The Business Meeting was followed by a Scientific Meeting, at which the following films were shown:

- (1) 'The science of milk production' (Dr W. E. Petersen, University of Minnesota, U.S.A.).
- (2) 'The *Xenopus* test for pregnancy (Messrs Ciba Ltd.).
- (3) 'Human sperm abnormalities' (Messrs Ciba Ltd.).





# THE JOURNAL OF ENDOCRINOLOGY

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## THE FOUNDATION OF THE SOCIETY FOR ENDOCRINOLOGY

On 15 February 1946 a meeting of twenty-two contributors to the *Journal of Endocrinology* unanimously resolved to form a society of the same name, and to invite as foundation members all others who had published in the *Journal*. A working committee was appointed to draft a constitution for the Society. This was adopted, after certain amendments, at the inaugural meeting of the Society, which was held at Guy's Hospital on 26 April 1946. At this meeting the following were elected to act as officers until the first annual general meeting:

<i>Hon. Secretary</i>	Dr S. J. FOLLEY
<i>Hon. Treasurer</i>	Dr C. W. EMMENS
<i>Hon. Editor of the Society's Proceedings</i>	Professor S. ZUCKERMAN
<i>Members of the Committee</i>	Dr P. M. F. BISHOP
	Dr A. S. PARKES
	Mr P. C. WILLIAMS
	Professor F. G. YOUNG

Apart from an annual general meeting for transacting the business of the Society, it was resolved that ordinary meetings at which original communications would be presented, and symposia on specific topics in the field of endocrinology, were to be arranged.

The first Annual General Meeting of the Society was held on 24 July 1946 at Guy's Hospital. Dr A. S. Parkes, F.R.S., was in the chair and the following were elected to serve as officers until the second Annual General Meeting:

<i>Chairman</i>	Dr A. S. PARKES
<i>Secretary</i>	Dr S. J. FOLLEY
<i>Treasurer</i>	Dr C. W. EMMENS
<i>Editor of the Society's Proceedings</i>	Professor S. ZUCKERMAN
<i>Members of the Committee</i>	Dr P. M. F. BISHOP
	Dr C. H. GRAY
	Mr P. C. WILLIAMS
	Professor F. G. YOUNG

The inaugural address, which appears below, was given by Dr C. R. Harington, F.R.S., who was introduced by the Chairman. A vote of thanks was proposed by Professor C. Rimington, and carried with acclamation.

## THE SCIENTIFIC FOUNDATIONS OF ENDOCRINOLOGY

## INAUGURAL ADDRESS TO THE SOCIETY FOR ENDOCRINOLOGY

By DR C. R. HARRINGTON, F.R.S.

When I received the invitation to deliver this inaugural address I was naturally much gratified by the honour which the Committee of the Society for Endocrinology had paid me, but at the same time I was somewhat surprised at their choice. Although it happens that much of my work has been concerned with one small aspect of endocrinology, I have always liked to regard myself as a biochemist; I certainly cannot pretend that it was a primary interest in endocrinology which led me to start work on the thyroid gland, and the mere fact that I have studied in some detail the hormone produced by this organ scarcely seemed to me to constitute a claim to the status of endocrinologist. Such reflections having led me to give some thought to the origins and nature of the subject of endocrinology and to the scientific qualities and acquirements which are necessary to make a well-equipped endocrinologist, it seemed to me that these themes might be worth developing on the present occasion.

Under present-day conditions, when many of us find it necessary to belong to more scientific societies than we care to think of, the formation of yet another such society is not a matter lightly to be undertaken; it can only be justified indeed if the branch of knowledge with which it is to deal has reached a stage of development disproportionate to the attention which can be given to it by existing bodies, and the first question for us is whether this stage has in fact been reached by endocrinology. This is a question which arises every time that investigators become sufficiently interested in the borderland between two or more existing branches of scientific knowledge to develop new techniques and new modes of thought, sharing something indeed of each of the disciplines from which they are derived, but eventually acquiring an individual quality of their own. It is after all not so long since the subject of biochemistry itself, the scientific independence of which is now universally recognized, was regarded as an offshoot or even as a poor relation of physiology, and even now the term biochemistry has some strange connotations. There are still some organic chemists who would claim that if they were only to go through the process of isolation of a natural product before embarking on its chemical investigation they would thereby constitute themselves biochemists. At the other extreme there are biologists who consider themselves or are considered by their biological colleagues to be biochemists so soon as they have occasion, perhaps with some fumbling and uncertainty, to employ a chemical technique in the course of their work. In spite of these persisting oddities, however, biochemists themselves and most other scientific men have a reasonably clear picture in their minds of the range of thought and activity which is covered by biochemistry, and an equally clear conviction of the justice of the claim of the subject to be regarded as an independent branch of science. The fact that we are gathered here to-day is proof that we are likewise convinced that endocrinology has now in its turn earned independent scientific status. If biochemistry, however, with its relatively clear and simple development from the

interplay of chemistry with the individual branches of biological science, has not easily or quickly established itself as an independent scientific discipline, we have to recognize that the corresponding difficulty may be even greater for endocrinology, the origins of which are far more complex. It may repay us therefore to consider for a short time what are the true foundations of endocrinology, and how it is that this subject has emerged from being one of the more obscure branches of physiology to the position of importance which we now believe it to occupy.

It seems to me that there is a curious contrast between the ways in which scientific knowledge is developing and scientific advances are being made on the physical and biological sides respectively. On the one hand the growth in complexity of each individual branch of physical science is such that the man who would master any one of these branches is necessarily confined ever more closely to his own field; he must in fact be subject to the criticism that he tends always to know more and more of less and less. Fifty years ago, for instance, a well-informed chemist could claim with justification to know the whole of the subject; nowadays, if we consider one division of the subject alone, there are few organic chemists who are completely at home in all branches of organic chemistry, and it is certainly not expected that organic and physical chemists should have much detailed knowledge of each other's fields of work. In the biological sciences on the other hand a stage has been reached in which no major advance is likely to be made except by a man who is something of a jack-of-all-trades, at least to the extent that he can appreciate and utilize some of the technique and much of the mode of thought of physics and chemistry, and often of mathematics. Of no field of biological endeavour is this more true than of endocrinology, and I am convinced that a continued recognition of this truth is the first essential for the further satisfactory development of the subject.

If I am to illustrate the point which I am trying to make it can only be by reference to that branch of endocrinology with which I can claim some personal acquaintance, namely the study of the thyroid gland. For the present purpose indeed this subject offers certain advantages, particularly in that the history of its development has been fully studied and can be traced over a long period, so that it is possible now to assess the contributions of different branches of science on which our present knowledge rests.

The earliest sources of information relating to the thyroid gland are scarcely to be described as scientific at all. They consist of repeated references to the commonest disease of the thyroid, namely simple goitre, which was of course not recognized as a malady of the gland itself but only as a swelling of the neck which had the characteristics of an endemic disease. Curiously enough most of the classical and early mediaeval references to goitre, particularly those calling attention to its endemic occurrence in certain types of locality, do not appear in the writings of medical men, but in those of general observers interested in natural history. In later mediaeval times the medical authors did indeed begin to give more detailed descriptions of the disease and to pay more attention to its peculiar geographical distribution, but they remained not unnaturally preoccupied for the most part with methods of treatment, still, be it noted, without association of the disease with a definite organ of the body. Indeed, by the time that the thyroid gland was first anatomically described by Wharton in 1656, a very considerable mass of information about simple goitre had

been acquired; remedies in great numbers had been proposed, and lest we should be inclined to despise the empirical method too thoroughly we may note that the remedy to which most consistent attention was paid proved, as we shall see, to be highly significant in the light of later developments.

Although Wharton's anatomical description of the thyroid gland emphasized the salient features of the organ, particularly its abundant blood supply and the presence in its follicles of a curious and characteristic colloid material, the influence of his contribution was surprisingly small in its immediate effect on the advance of knowledge of the function of the gland and the nature of the diseases. Suggestions with regard to thyroid function were indeed made on the basis of anatomical observation, such as that the colloid substance of the gland was a lubricant for the trachea, and that the gland formed a vascular shunt which regulated the supply of blood to the brain, but these were no more than guesses and in no case was any experimental evidence forthcoming in their support. In the event it was actually 200 years after the anatomical identification of the thyroid gland by Wharton before this gland was proved to play any essential part in the normal economy of the body. In the meantime, however, other branches of investigation had been pursued with profit and in particular great advances had been made in the understanding and treatment of simple goitre, which from the time of Wharton was recognized in its true colours as a pathological enlargement of the thyroid gland.

I have already referred to the fact that there was one therapeutic remedy for goitre which was described so consistently by all the early medical writers as to merit the assumption that it must have met with some measure of success; this was the remedy of burnt sponge. The administration of the ash of sponges is said, I do not know on what authority, to have been recommended for the treatment of goitre by Chinese physicians about 2000 B.C. In more recent times the prescription appeared first, so far as I know, in the writings of Arnaldus in the thirteenth century, and was repeated by almost every continental medical author dealing with the subject up to the end of the eighteenth century. Another method of treatment for which success was claimed frequently, although not with such consistency as in the case of burnt sponge, was the administration of sea-water. These observations remained entirely empirical in character until the early years of the last century when they suddenly received a completely reasonable explanation, which came about through the impact of the work of two chemists on the mind of a physician. The physician was Coindet of Geneva and the chemists were Courtois, who was a manufacturer of salt-petre, and Gay-Lussac. The story of the events which led to the isolation of iodine from the ash of burnt sea-weed by Courtois and its identification by Gay-Lussac as a new element is well known; the inspiration of Coindet that this element newly discovered in a marine organism might be the active principle of the time-honoured burnt sponge remedy for goitre represents in my opinion one of the outstanding advances in the study of the thyroid gland, and indeed, in view of later events, in the study of endocrinology itself.

The initial successes which Coindet achieved in the treatment of goitre with iodine were extremely dramatic and attracted widespread attention, but they did not, as might perhaps have been expected, lead to a direct association of iodine with the normal thyroid gland. Although it may now seem surprising that the possibility of

such an association did not occur to anyone, we have to remember that at this time there was still no knowledge of normal thyroid function and that the gland was regarded more generally as the seat of a pathological process than as an essential organ of the body. Apart from its intrinsic importance, however, Coindet's work made possible the correlation on a reasonable basis of the great mass of geographical and geological information which had been accumulated in the course of centuries on the subject of the distribution of goitre.

As exploration of the world had extended it had become more and more evident that the regions in which endemic goitre might be expected were those which were mountainous or far removed from the sea, or both. Thus in Europe the Alps, the Carpathians and the Pyrenees were the main centres of goitre; in Asia the disease was found in Turkestan and in the Himalayas; in North America it prevailed in parts of the Rocky Mountain area and in the districts surrounding the Great Lakes; in South America the Spanish colonists found it in the Andes, and so on. It could thus be said that there was a negative correlation between the occurrence of goitre and the only source known at that time from which the specific curative agent discovered by Coindet could be obtained in quantity, namely the sea.

It was evidently reflection on these facts which led to the next contribution to the goitre problem. This came again from a chemist; it consisted of the wide survey which was carried out in France in the middle of the last century and which had for its object the study of the relationship, if any, of the occurrence of endemic goitre to the iodine content of the water drunk by the population. Chatin was in fact able to show in a large number of cases that severe endemic goitre was associated with a water supply of very low iodine content, whilst the disease was hardly ever to be found where the water was relatively rich in iodine. In retrospect the results obtained by Chatin seem to be entirely convincing; at the time they attracted sufficient attention to be made the subject of enquiry by a special Commission appointed by the French Academy of Sciences, but this Commission found the evidence unsatisfactory and pronounced against the idea that there could be a causal relationship between a disease of the thyroid gland and alimentary intake of iodine. It is perhaps not very surprising that this view should have been taken; there were a few anomalous results among Chatin's own findings, and it was obviously easier for a body of men labouring under the weight of medical tradition to seize on an excuse to dismiss his work than to accept a revolutionary idea. The notion that a disease could be caused, not by an actively noxious agent, but by the mere deficiency of an element which in any case was never present except in minute quantities, was one for which medical and scientific opinion was quite unprepared. The result was that Chatin's conclusions were only vindicated many years later, when other lines of evidence induced chemists to return to the question and, by results obtained in many different countries, to place the matter beyond doubt.

The chemists having thus been brought to a temporary halt, it was again the turn of the medical men to make an advance, and this was not long in coming. In 1873 the first description was published by the English physician Gull of a disease characterized mainly by thickening of the skin, loss of hair, increase of weight and retrogression of mental faculties; soon afterwards two more such cases were described by Ord, who named the condition myxoedema: moreover, in one of these cases which



came to post mortem he discovered that the thyroid was atrophied. Here then was the first suggestion of a really important function for the normal thyroid gland, although it could not be claimed with certainty that the atrophy of the thyroid was the sole cause of the myxoedematous condition. Confirmation of the validity of this suggestion was rapidly forthcoming from the observations of certain Swiss surgeons, particularly Kocher. The medical treatment of simple goitre with iodine which had been initiated by Coindet had, after its early successes, fallen into some disrepute owing to the toxic effects which were produced by overdosage, and in consequence the treatment of choice in Switzerland for simple goitre had become surgical excision of the enlarged thyroid. Usually such excision did not involve the complete removal of all thyroid tissue, but in a few cases, owing to the severity of the pressure symptoms which had been caused by the goitre, radical excision of the entire swelling was carried out. Kocher observed that cases which he had treated thus showed the immediate improvement which was expected, but in the course of a few weeks or months began to show symptoms which he recognized to be identical with those of the recently described myxoedema. These observations of Kocher, soon confirmed by other surgeons, and taken in conjunction with the work of Gull and Ord, proved beyond doubt that the thyroid gland had indeed an essential function to perform and the way was paved for experimental physiologists to proceed with the analysis of its role in the normal economy of the body. The issue of that part of the early physiological work which depended on the observation of the effects of thyroidectomy in normal animals was somewhat confused by failure to recognize the parathyroid glands; nevertheless, the main outlines of the normal role of the thyroid gland were soon established.

From the proof of the essential nature of the thyroid gland to the treatment of naturally occurring thyroid deficiency, as manifested in myxoedema, by replacement therapy, seems to us now to be but a short step; actually it was some years before this step was taken. The first attempt consisted of the subcutaneous implantation of normal thyroid tissue from an animal into patients suffering from myxoedema in the hope that a permanent graft might be established; naturally such foreign tissue was rapidly absorbed, but it was noticed that during the process of absorption the symptoms of the disease were much alleviated. This observation supplied the hint which induced Murray to make his famous therapeutic experiment of treating a myxoedematous patient with injections of a crude extract of thyroid gland, an experiment which proved that such a patient could be restored to and maintained in a normal condition so long as the exogenous supply of thyroid substance could be continued. The inconvenience of repeated injections of crude tissue extract soon led to attempts to substitute treatment by oral administration of thyroid gland and the success of these attempts laid the foundations of replacement therapy of myxoedema as it is practised to this day. I think it is fair to say that in the identification of myxoedema with a condition of thyroid deficiency, followed by the successful treatment of this condition by administration of thyroid substance, we have the real foundation of modern endocrinology. These two discoveries proved in the first place that the thyroid gland was an essential organ, and in the second, since it had no duct, that it must exercise its effect through the medium of a substance passed directly from it into the blood stream; they proved in fact that it was an organ of internal secretion.

It was natural that the proof that the thyroid gland performed its function by the secretion of an active principle should arouse speculations as to the nature of this principle; the time was in fact now ripe for a chemical examination of the thyroid gland itself, and such an examination was undertaken with a considerable measure of success. It would be pleasing for me as a biochemist if I could claim that at this stage, on the threshold of the first great biochemical contribution to the thyroid problem, it was a biochemist who received inspiration. This was, however, not the case; it was the surgeon Kocher who, reflecting on the recent discoveries concerning thyroid function and recalling the earlier well-established therapeutic effect of iodine in goitre, suggested that a search might profitably be made for iodine in the thyroid gland itself. Under the direct inspiration of Kocher this search was carried out by Baumann, who was soon successful in finding the element which he was seeking. Baumann did not stop with the mere demonstration of the presence of iodine in the gland; he showed that it occurred in organic combination and that it was associated with, if not actually a component of, the physiologically active principle.

This early and fundamental biochemical attack on the thyroid gland was pursued by Baumann himself and others, notably Oswald, in the direction of attempts to concentrate and purify the organic iodine-containing components of the gland; these experiments confirmed Baumann's suggestion of the direct association of iodine with physiological activity, but did not lead far towards the isolation of the pure active principle, and it was twenty years before biochemistry could again offer a significant contribution. In the meantime, however, an advance had come from an entirely different quarter, namely that of experimental pathology. It had been shown some years previously by Halsted of Baltimore that after removal of five-sixths or more of the thyroid gland of a normal dog the residual fragment of thyroid tissue underwent a characteristic series of changes in which there was a great hyperplasia of the glandular epithelium accompanied by disappearance of colloid from the follicles; this was regarded not unreasonably as a histological picture of a compensatory process of over-activity on the part of the small remaining thyroid fragment. A little later Marine, at that time in Cleveland, Ohio, took up the histological study of the goitre which commonly occurred in dogs in the endemically goitrous area where he was working, and he observed among these glands a number which showed histological changes precisely similar to those which had been described by Halsted as the result of sub-total thyroidectomy. This was in itself an interesting observation, but Marine's outstanding contribution came from the fact that he had the wit to combine his histological studies with chemical analysis of the glands; in this way he was able to establish the important generalization that normal histology of the thyroid gland implied a certain minimum concentration of iodine in the tissue. If, through deficiency of iodine intake, the iodine concentration in the thyroid tissue fell below the critical value signs of glandular hyperplasia made their appearance, and the severity of these signs was closely proportional to the deficiency in concentration of iodine. Marine explained his results by application of the argument employed by Halsted in respect of sub-total thyroidectomy. He regarded the glandular hyperplasia as a work hypertrophy of a gland struggling against adversity which was caused not, as in the case of Halsted's dogs, by loss of a major part of the gland substance, but by inadequacy of the supply of an essential component of its active secretion. Although

Marine's hypothesis cannot now be accepted, a more reasonable explanation of his (and incidentally of Halsted's) results being available through modern knowledge of anterior pituitary-thyroid relationships, there is no doubt that his generalization regarding the relation of iodine to pathological thyroid enlargement was an achievement of the first magnitude. It certainly stimulated the renewed studies by such men as McClendon in America and von Fellenberg in Switzerland of the relationship of environmental iodine deficiency to endemic goitre, which not only finally vindicated the work of Chatin, but led to the widespread adoption of an important measure of preventive medicine in the shape of the iodine prophylaxis which is now commonly practised in goitrous regions. It is probable that Marine's work also encouraged the renewed biochemical attack on the nature of the active principle of the thyroid gland which culminated in the isolation of thyroxine by Kendall of the Mayo Clinic.

Kendall's great achievement was a major biochemical contribution in that it settled once for all the nature of the association of iodine with the physiological activity of the thyroid gland, an association of which the existence had been indicated by so much previous work; it also made possible physiological experiments of greater precision than could formerly have been undertaken. In itself however it did not do much to advance the study of the nature and mode of action of the active principle; such an advance had to await the development of an improved method of isolation of thyroxine and the study of the chemistry of the compound. It was at this stage that work on the problem was begun in my own laboratory and by a combination of biochemical and chemical modes of attack we were able to devise a method by which adequate amounts of thyroxine could be isolated from the thyroid gland, and then to proceed with the determination of its constitution and ultimately with its synthesis. The elucidation of the chemical structure of thyroxine immediately suggested the idea that the compound was probably formed in the body from tyrosine through the stage of diiodotyrosine, and this biogenetic hypothesis actually played an important part in determining the plan of synthesis which was eventually successful. It was not long before the idea received further strong support by the isolation of diiodotyrosine from the thyroid gland and the production of evidence that the whole of the thyroid iodine could be accounted for by summing up the respective iodine contents of the diiodotyrosine and thyroxine which it contained. Moreover, the isolation by enzymic hydrolysis of thyroid gland of natural thyroxine in the optically active form, made possible the chemical study of the configurative relationship of this compound to natural *L*-tyrosine; the proof which was obtained that the two compounds had in fact the same configuration was consistent with the idea that one was derived biologically from the other.

Further evidence bearing on this point came with the discovery in Germany just before the war that iodination of proteins unrelated to the thyroid gland could give rise under suitable conditions to products which had the characteristic physiological activity of the thyroid gland and from which thyroxine could be isolated. This discovery carried with it the implication that the direct oxidation of diiodotyrosine to thyroxine by chemical means was a possibility, and such a possibility has now been demonstrated. In my own laboratory we were able to obtain yields of thyroxine of 4-5 % by the oxidation of diiodotyrosine with hydrogen peroxide, and we have also shown that the same oxidation, though in less good yield, can be brought about by

iodine itself; it is therefore apparent that, in so far as purely chemical methods are capable of settling the matter, the mode of biosynthesis of thyroxine was established. It only remained to demonstrate the actual process in the living thyroid tissue, and even this has recently been accomplished in America by calling in the resources of still another branch of science, namely nuclear physics. With the aid of a radioactive isotope of iodine it has been possible to observe the transfer of iodine, introduced into the body as iodide, through diiodotyrosine into the molecule of thyroxine; moreover the series of reactions can be followed not only in the intact thyroid gland but also in slices of thyroid tissue surviving *in vitro*. More surprisingly, experiments with radioactive iodine have even revealed the possibility of extra-thyroidal formation of thyroxine in the body; this observation is of the first importance in relation to the process of biosynthesis of thyroxine; taken in conjunction with the purely chemical work on the formation of thyroxine from diiodotyrosine which I have already described, it enables a clear picture to be formed of the actual biochemical mechanism of the synthesis of thyroxine *in vivo* and of the true role of the thyroid gland in relation to this process. I believe, indeed, that we may now conclude that the essential biochemical step in the biosynthesis of thyroxine is the liberation of iodine from iodide by an enzymic oxidizing system. If this event occurs in a living tissue all the conditions are fulfilled which we know to suffice for the formation of thyroxine *in vitro*; we have tyrosine, which is a constituent of all body proteins, and we have iodine in a form in which it is able both to produce diiodotyrosine from tyrosine and also to oxidize the former to thyroxine. Should this conception be correct we may further conclude that the essential function of the thyroid gland consists in its specific capacity to accumulate iodine in high concentration.

For simplicity's sake I have departed from chronological order in completing the story of the work on the chemistry of thyroxine and on its biosynthesis to the point at which we have arrived to-day. It must be remembered, however, that the burst of biochemical activity in research on the thyroid gland, which began with Kendall's isolation of thyroxine, was accompanied by many physiological, or perhaps I should at this stage say endocrinological investigations, some of which have proved to be highly important. Since the serious physiological study of the organs of internal secretion began, the question of the interrelations between these organs is one which has constantly exercised the minds of investigators. In the early days, before any adequate experimental evidence had been acquired, much nonsense was talked and written about the endocrine balance of the body. After many false starts, however, some solid facts began to be established and among these was that of the production by the anterior pituitary gland of a hormone which could stimulate the activity of the thyroid gland. I believe I am right in saying that the discovery of the thyrotrophic hormone of the anterior pituitary gland was the first real indication of the unique controlling function of this gland in relation to the rest of the endocrine system. The later discovery of the balance existing between the anterior pituitary and thyroid glands, through which the output of anterior pituitary thyrotrophic hormone is itself regulated by the concentration of circulating thyroid hormone, has provided, as I have already indicated, a reasonable explanation of the effects observed by Marine in conditions of iodine deficiency. It is clear that if iodine deficiency be prolonged there will necessarily be an eventual reduction in the amount of thyroxine

produced by the thyroid gland; when this reduction has proceeded so far as to cause a diminution in the concentration of circulating thyroid hormone the output of thyrotrophic hormone will be increased and the thyroid gland will be stimulated to greater activity, which will in turn be reflected in the glandular hyperplasia which Marine observed. This hyperplasia, therefore, is not to be regarded as a primary response of the thyroid gland to deficiency of iodine but as a secondary phenomenon resulting from disturbance of the normal regulating mechanism.

There remains one further aspect of modern research on the thyroid gland to which I should like to refer, namely that which is concerned with thyroid inhibitors, the term being used to signify substances which interfere with the functional effects of the thyroid gland in the body. Interest in such compounds originated in the observation of the goitrogenic effect of a cabbage diet in rabbits; analysis of this effect led to the discovery of the goitrogenic action of cyanates, thiocyanates, nitriles and some related compounds. The changes induced in the thyroid gland by this group of substances consisted in gross enlargement with a high degree of glandular hyperplasia, changes precisely similar in fact to those observed by Marine in iodine-deficient animals; moreover, the goitrogenic effect of the compounds could be neutralized by the simultaneous administration of adequate amounts of iodine. The deduction was made therefore that goitrogens of this type exercised their effect by interfering with access of iodine to the thyroid gland and by thus producing a state of conditioned iodine deficiency in animals receiving a diet which would normally be adequate in respect of its iodine content. In quite recent years a second group of thyroid inhibitors has been discovered, the members of which, typified by thiourea, have the same effect on the thyroid gland; in this case however the effect cannot be overcome by simultaneous increase in intake of iodine, and it has therefore been assumed that the essential action of thiourea and its congeners is to interfere with the biochemical process in the thyroid gland leading to the formation of thyroxine. It is clear that the physical effects of both types of goitrogens on the thyroid gland itself are just those to be expected from our knowledge of the anterior pituitary-thyroid relationship. By making use again of the radioactive isotope technique it has been possible to examine the validity of the deductions which I have mentioned regarding the details of the biochemical mechanism by which the process of inhibition is brought about. Experiments of this type have shown quite clearly that inhibitors of the cyanate group do in fact prevent the access of iodine to the thyroid gland, whilst those of the thiourea group, although they have perhaps some slight effect of the same sort, act principally by blocking the synthetic process within the gland itself. There is evidence that this blocking is brought about by inhibition of an enzymic reaction, in all probability the oxidation of iodide to iodine which, as I have indicated, is in my opinion the essential biochemical step in the natural formation of thyroxine.

This brings me to the end of the brief outline of the history of work on the thyroid gland which I have chosen as an illustration of my theme to-day. I have left many gaps in the story; for instance I have omitted all reference to such perplexing problems as that of Graves' disease. From what I have had time to say, however, I think that it will be clear that so far as this particular problem of endocrinology is concerned we have accumulated a very considerable body of information. There is much more that we should like to find out and which in time we doubtless shall find

out; even now however we know the chemistry of the active principle of the thyroid gland and much concerning its physiological action; we understand its biosynthesis, and the acquisition of this knowledge has involved the study and explanation of the mode of regulation of the activity of the gland itself both in normal and abnormal conditions; we have, moreover, learnt methods of controlling this activity which may be said to have a truly physiological basis.

I hope I have also succeeded in making it clear that we owe this large amount of information about the thyroid problem to the interplay, over a long period of time, of many different kinds of scientific effort. The story which I have told has dealt with the contributions of natural historians and explorers, geographers and geologists, physicians and surgeons, biologists and pathologists, chemists, biochemists and physicists; what branch of science is unrepresented? In this respect the history of study of the thyroid gland is surely an admirable example of the multiple scientific foundation of endocrinology and of the interweaving of scientific disciplines which is essential to its success. From another point of view however this history may serve us as an object lesson, for it must make us realize that if all the branches of science involved had been used to the best effect it would scarcely have taken us some thousands of years to reach our present imperfect state of knowledge of the thyroid gland. Many times progress has been arrested by the weight of traditional authority and by lack of mutual appreciation among different scientific workers; the prejudice of the French physicians and their suspicious attitude towards chemists delayed the recognition of the aetiology of simple goitre by half a century, and if it had not been for their ignorance of biology the chemists would certainly have been quicker to derive inspiration from the observations of the physicians and surgeons. I am convinced that it is of the first importance for endocrinologists to bear these past events in mind, satisfied though they may be that the present tendency is much more favourable.

A writer in the *Lancet*, commenting recently on the much to be welcomed formation of a Section of Endocrinology in the Royal Society of Medicine, remarked that 'the [clinical] endocrinologist must be more than a clinician with a slant; he must... be something of a biologist, something of a biochemist and everything of a physiologist'. I would rather say that an endocrinologist must be everything of an endocrinologist; by this I mean that he should be a person whose chosen field of work is the study of the organs of internal secretion, who is prepared to develop his own new techniques appropriate to his special work and who is also prepared to draw upon the resources of any other branch of science when the need arises. No endocrinologist can hope to know, in the true sense, all the sciences on which his own work rests, but he must know his way about if he is to find his way home; he must have a lively appreciation of the contributions which different techniques can make to his own particular line of effort and he must be ready to receive inspiration from whatever quarter it may come, whether as so often in the past from the observations of his clinical colleagues, or from the fundamental studies of the biologist and the biochemist. I suggest that if the members of this Society approach their work in this way there is no doubt that endocrinology will flourish in their hands. As it flourishes, so will new and valuable recruits be attracted by the realization that endocrinology offers a field of work worthy of any man's endeavour, and so will come about the growth and development of endocrinology, the promotion of which is the principal aim of our Society.

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The following demonstrations were also given:

On some effects of the combined gonadotrophic hormones. By P. BACSICH, A. SHARMAN and G. M. WYBURN

Varying doses (from 1 to 15 Synergy rat units) of combined gonadotrophic hormones (Synapoidin) were administered subcutaneously to immature female guinea-pigs (2 months old). Contrary to expectations, only luteinization was observed. The extent of luteinization was proportionate to the dose. In some cases, after large doses, luteinization involved practically the whole ovary, so that even to the naked eye the ovary appeared as one large corpus luteum. On the basis of histological analysis, it is believed that the lutein tissue is of follicular origin.

Results of the experiment were demonstrated by colour photo-micrographs.

Steroid anaesthesia. By C. W. EMMENS

The anaesthetic activity of various cyclopentano-perhydrophenanthrene derivatives was discovered by Selye (*Proc. Soc. Exp. Biol. Med.* 46, 142, 1941). This property is apparent only when they are administered by a route which ensures rapid absorption, such as intravenous injection in propylene glycol or intraperitoneal injection in oil.

The partially hepatectomized female rat weighing about 50 g. was found to be a sensitive test-object, presumably because the steroid compounds are normally inactivated by the liver. The dose of some of the most active compounds required to cause loss of the righting reflexes in the majority of such animals is reported by Selye as follows:

Pregnanedione	0.8 mg.	Dehydrocorticosterone	3.5 mg.
Desoxycorticosterone acetate	1.0 mg.	Acetoxypregnenolone	4.0 mg.
Progesterone	2.0 mg.	Androsterone	4.5 mg.

Practically all steroids which exhibit hormone-like activity of one type or another also produce anaesthesia when given in large enough doses, but maximal potency is associated with saturation of the molecule or the presence of only one double bond, and with terminal oxygen atoms. No oestrogen, for instance, is a very potent anaesthetic.

In the present demonstration, immature male and female rats have received desoxycorticosterone acetate or progesterone by the intraperitoneal route. Males are usually less sensitive than females; the dose of desoxycorticosterone acetate required to produce surgical anaesthesia is about 0.2 mg./g. in males and 0.1 mg./g. in females, but individual variation is large. The anaesthesia is slow in onset and lasts for several hours. Progesterone has approximately half the potency of desoxycorticosterone acetate.

It is of interest to compare these anaesthetics with Nembutal, which produces anaesthesia of about the same duration:

Substance	Dose required for surgical anaesthesia in immature rats (mg./g.)	
	Male	Female
Nembutal	0.05	0.05
Desoxycorticosterone acetate	0.2	0.1
Progesterone	0.4	0.2

Modifications of development in *Discoglossus* tadpoles. By H. M. BRUCE

Under laboratory conditions at water temperatures of 20–22° C., normal *Discoglossus* tadpoles reach metamorphosis at about 4 weeks of age, when the total length of the tadpole is about 28 mm. In this species metamorphosis is rapid and regular.

The addition of thiourea at a concentration of 1 : 1000 to the water in which the tadpoles are kept inhibits metamorphosis but is without effect upon growth as measured by total length. Under these conditions the animals continue to grow and will eventually attain a total length of nearly 60 mm., which represents a relatively much greater increase in total size. At lower concentrations of thiourea metamorphosis is delayed but not completely inhibited and the resulting toads are larger than the normal toads at metamorphosis.

The addition of stilboestrol in doses ranging from 1 : 2,000,000 to 1 : 20,000,000 has no influence upon the inhibiting action of thiourea nor upon growth rate on the ultimate maximum size attained.

The addition of growth hormone in the form of fresh ox anterior pituitary given as food in place of the rabbit or guinea-pig liver generally used, was also without influence upon the inhibiting action of thiourea, but the tadpoles so fed did not grow so well nor attain quite the same maximum size as those fed on liver.



# PROCEEDINGS OF THE SOCIETY FOR ENDOCRINOLOGY

## SYMPOSIUM ON THE ANTERIOR PITUITARY-GONADAL RELATIONSHIP

*Held at The Royal Veterinary College, Camden Town, London,  
on 12 December 1946*

Dr P. M. F. BISHOP, who was in the Chair, opened the proceedings by welcoming to the Society Prof. R. Courrier of the Collège de France, Paris, Dr C. Hamburger of the State Serum Institute, Copenhagen, and Dr M. Klein of the University of Strasbourg, and by thanking the British Council who had made their visit possible.

### **Pathways in the environmental stimulation of the pituitary.**

By Prof. S. ZUCKERMAN. *Department of Anatomy, University of Birmingham*

In most animal species breeding is confined to a specific season of the year. Breeding seasons vary with different animal types and according to environmental conditions, but in general most species in their normal habitats mate at a time, taking into consideration the period of gestation, which is favourable for the well-being of their offspring.

With only a few known exceptions, an animal belonging to a species indigenous to the southern hemisphere adjusts its breeding season to the new calendar when it is moved to the northern, so that within a year or two the time of anoestrus in the southern hemisphere coincides with the new breeding season in the northern. Baker's (1939) studies of birds have shown, moreover, that as one moves north from temperate latitudes, one finds a general tendency for the egg-laying season to start later and later at the rate of some 20-30 days per 10° latitude.

In analysing the factors that control the periodicity of breeding we may start from the firmly established fact that the definitive changes in the reproductive organs which determine the psychological and physiological aspects of the breeding behaviour of animal species are conditioned by the internal secretions of the gonads, and that the gonads are predominantly under the control of the anterior lobe of the pituitary.

In some way, therefore, the pituitary gland is a mediating structure through which the organism adjusts its reproductive life to environmental change. Considering the problem *a priori* the factors which may be concerned are:

- (a) changes in the duration, intensity and type of light,
- (b) changes in temperature,
- (c) changes in humidity and rainfall,
- (d) changes in the nutritional environment.

Information on the subject is available for amphibia, birds and mammals. Savage [1935], for example, has made an exhaustive study of the migration and spawning of the common frog. His observations in general confirm the long-standing view that rainfall has a considerable influence on the timing of the breeding season of Amphibia.

Thus the migration of frogs to the ponds in which they breed is due to specific olfactory stimuli emanating from the ponds themselves, and which are immediately due to certain chemical products of their algal fauna. Spawning does not begin until a further change in the algal cycle again changes the chemical constitution of the water. This change, Savage suggests, is perceived by the animals through skin receptors and acts on the gonads *via* the pituitary.

The algal cycle which determines the breeding cycle is affected by the previous rainfall, and by the phosphate content of the ponds. The more rapidly the phosphate is used up by the algae, the sooner the frogs spawn. Spawning is more likely to occur in wet weather, but its mean date is influenced by the weather for a relatively long preceding period.

The annual cessation of ovarian activity in another amphibian, *Xenopus*, is due to the seasonal decrease in the water volume of the ponds it inhabits [Alexander & Bellerby, 1938]. Bellerby [1938] has also shown that the sexual cycle is not influenced by seasonal variations in light intensity or wave-length. By exclusion, he concludes that the sexual cycle of *Xenopus* is related to changes in water volume and to the effects of temperature and nutrition.

The environmental stimulant of the pituitary which has been most studied in birds is light. Encouraging egg production by subjecting hens to extra illumination is a very old practice. But it was only in 1925, when Rowan showed that the parallelism of increased gonad size, increasing hours of daylight and northward migration in the *Junco* was no accident, that the problem became one of major scientific interest. What Rowan essentially found was that while food supply, weather conditions, temperature, barometric pressure, etc., might have some secondary influence on migration, its proximate stimulus was the waning and waxing of the days. Rowan later suggested that not light stimulation *per se*, but increased exercise was the proximate cause of sexual recrudescence. Most workers, however, do not agree with him in attributing such importance to increased exercise.

His observations on *Juncos* have been confirmed on many species, and his experimental studies on birds have been much extended by Bissonette and Benoit. Bissonette, for example, found red light a more effective stimulator of the gonads than green. He also developed the thesis that intensity of light was an important factor. Benoit, working on ducks, has also shown that light is the factor that stimulates gonadal growth, red rays having the maximum effect. He further found that the response is not elicited in the absence of the pituitary, or in normal birds that are hooded. Conversely, he has demonstrated that it occurs when the hoods are provided with holes for the eye regions, and when the optic nerves are cut or the eye-balls completely removed. The inference that light acts directly on the pituitary through the ocular region was substantiated in experiments in which light was directed on to the gland through a quartz rod placed in the empty orbital cavities. Another of Benoit's contributions was to show that there is a quantitative relation between the gonadal response of drakes and the duration and intensity of illumination.

Seasonally breeding mammals show the same sensitivity to light, although positive results have not been obtained in some species, for example, the guinea-pig [Dempsey, Myers, Young & Jennison, 1934] and the spermophile [Moore, Simmons, Wells, Zalesky & Nelson, 1934].

Anoestrous ferrets, as Bissonette [1932] first showed, become reproductively active in winter if the number of hours of light to which they are exposed daily is prolonged artificially. This response cannot be elicited after hypophysectomy [Hill & Parkes, 1933] and is much impaired by division of the optic nerves, or the deprivation of light, an observation which led Marshall & Bowden [1934] to propose that it is mainly dependent on the effects of stimulation on the pituitary. Marshall also showed that the effective range of light extended from the red as far as the ultra-violet, which was remarkably effective in prolonging the breeding season. The sensitivity of ferrets to light, and mainly to the factor of intensity, is greater in the female than in the male.

In the ferret the integrity of the optic nerves appears to be essential to the light-gonadal response, and the problem has been to discover where in the optic pathway, if anywhere, fibres diverge to affect the pituitary specifically. Experiments in which lesions were made in various places in the optic pathways failed to answer this question, but led to the conclusion that neither the visual cortex nor the midbrain is essential to the gonadal-light response in the ferret [Clark, McKeown & Zuckerman, 1939].

How the anterior lobe of the pituitary is stimulated by, presumably, nervous stimuli from the retina also remains a problem, since no one has yet been successful in showing that the pars glandularis, the source of the gonadotrophic stimulus, receives any significant innervation from the hypothalamic region, or that the latter has specific connexions with the retina. For the same reason it is difficult to explain as a specific neurohumoral reflex the occurrence of ovulation in rabbits after mating, for there is no evidence to show how the initial nervous stimulus of mating is transmitted to the pars glandularis itself, where it is transformed into the chemical one which affects the ovaries.

This part of the mechanism, the transformation of neural to humoral conduction, is a problem that badly needs elucidation.

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Dr G. W. HARRIS, in commenting on this last point, suggested that one link in the chain of events between the environmental stimulus and the gonadal reaction may be constituted by the hypophysial portal vessels. The nerve supply of the adeno-hypophysis is meagre, but the vascular link (in the form of the portal vessels) between the median eminence and the adeno-hypophysis is well developed in all forms so far studied. It seems possible, on the available evidence, that the nervous system regulates the activity of the adeno-hypophysis by means of a humoral relay through these vessels.

Remarking on this suggestion, Prof. Zuckerman replied that he did not feel it was sustained by adequate anatomical evidence, and that in his view much remained to be done before definite anatomical pathways could be posited. The crucial experiment of studying the effects of light in animals whose own pituitaries had been replaced by pituitary tissue transplanted to some extracranial site had not been carried out.

Dr REISS suggested that clinical experiments on patients might contribute to our conceptions of the neuro-anterior pituitary relationship. After prefrontal leucotomy, for example, the excretion of total 17-ketosteroids, oestrogens and adrenocorticotrophic hormone was increased. In several female patients where menstruation had ceased for many years, it started again after leucotomy, and soft atrophied testes of male patients were considerably improved after this operation. The existence of a centre in the frontal lobe which inhibits or controls the function of the pituitary anterior lobe is, therefore, not improbable.

**Oestrogens and gonadotrophic extracts.** By Prof. R. COURRIER.  
*Collège de France, Paris*

It is generally accepted that oestrogens promote the hypertrophy and persistence of previously formed corpora lutea, and some authors even claim that they also determine the formation of the corpus luteum. A question that must be answered is whether such effects occur directly or are mediated through the pituitary gland.

Williams [1940] and Pencharz [1940] have shown that oestrogens have a direct action upon the ovary and can bring about ovarian hypertrophy in the hypophysectomized rat. In such experiments the immature ovaries of 21-day-old rats increased from 7 to 28 mg. in weight, and were full of medium-sized follicles. The inference, here, is that a direct effect is concerned.

The combined effects of oestrogens and gonadotrophins on the ovary have also been the subject of much inquiry, but the results of work on rats and on rabbits are not in agreement. In the hypophysectomized rat, oestrogen seems to enhance the effect of gonadotrophin. Williams [1940], for example, found that the effect of serum gonadotrophin is increased by the simultaneous administration of diethylstilboestrol, and the same result was observed by Pencharz [1940] using chorionic gonadotrophin (PU). In the absence of the pituitary gland, PU alone stimulates only the theca of the follicles. If diethylstilboestrol is added, the response is considerably reinforced; the ovaries increase in weight, and contain numerous corpora lutea, large follicles and haemorrhagic follicles.

Corresponding results have been obtained by Simpson, Evans, Fraenckel-Conrat & Choh Hao Li [1941], who also report that a similar synergism exists between stilboestrol and the F.S.H. pituitary factor.

The effect of combined treatment with oestradiol and pituitary gonadotrophin has been studied by M. Aron and his co-workers on immature, but non-hypophysectomized rats. They found that a quantity of gonadotrophin which by itself produced only a weak response in the follicles had a luteinizing effect if its action was combined with that of oestrogen.

These different studies seem to show, therefore, that certain oestrogens reinforce the action of gonadotrophins upon the rat's ovary, whether or not the pituitary is removed.

A contrary conclusion emerges from the work of Dr E. Colombo on rabbits. His experiments appear to show that oestrogen counteracts the biological effect of gonadotrophins, and his results have been confirmed both by Ramos and by Fabiao, who consider the counter-action to be a fundamental mechanism in the maintenance of the hormonal equilibrium of the female sex cycle.

M. Fabiao injected rabbits weighing from 1200 to 1500 g. with 3000 i.u. of oestrone and 100 i.u. PMS. The usual ovarian response did not occur, whereas 50 i.u. PMS injected alone gave a marked reaction. The same result was also obtained with chorionic gonadotrophin.

Corresponding experiments which Prof. Courrier himself had carried out did not lead to conclusions as categorical as those of the South American authors. He obtained neither total absence of reaction, nor obvious synergism, when both oestrogen and gonadotrophin were given; and even after a long and intensive treatment with oestrogen, the ovary was still able to react to the chorionic gonadotrophin, even though the reaction seems to have been reduced in intensity.

Work carried out in collaboration with G. Gros, in which large doses of oestradiol and serum gonadotrophin were administered to monkeys, yielded corresponding results.

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In commenting on Prof. Courrier's paper, Dr BACSICH remarked that some years earlier Dr Folley and he had studied the effect of high daily doses of oestradiol monobenzoate on lactating rats. The corpora lutea of lactation were considerably enlarged, and furthermore, miliary corpora lutea frequently developed beneath the germinal epithelium, together with scattered luteal cells throughout the stroma. They had assumed that this apparent luteinizing effect is an indirect one mediated through the pituitary. If, on the other hand, Prof. Courrier's interpretation of the direct effect of oestrogens on the ovary is correct, views on pituitary-gonadal relationships would have to be revised.

More recent work (with Drs Sharman and Wyburn) showed that luteinization can occur without previous follicular stimulation. Thus the administration of combined gonadotrophins to immature female guinea-pigs causes only luteinization in the ovaries. Variations in the dosage produced only quantitative differences in the reaction.



**Anterior pituitary-like hormones.** By Dr I. W. ROWLANDS.\**Wellcome Veterinary Research Station, Frant, Sussex*

Gonad-stimulating substances may be divided into two groups according to their probable origin. The gonadotrophic hormones found in the urine after the normal cessation of gonadal function at the menopause, and after gonadectomy, are secreted by the pituitary gland. Those which occur in body fluids during pregnancy and in certain pathological conditions appear to have a different origin.

*Source of anterior pituitary-like hormones.* It is generally accepted that chorionic gonadotrophin is of placental origin. The earlier evidence for this view, the gonadotrophic activity of placental extracts [Collip, 1930], has been strengthened greatly by the *in vitro* cultivation of tissue from a 3-month human placenta [Gey, Seegar & Hellman, 1938; Jones, Seegar, Gey & Gey, 1943]. Cell-free filtrates of tissue cultured continuously for over 2 months possessed hormonal activity similar to that occurring in the urine of pregnant women. The components of the cultured tissue which showed most active growth were the cells of Langhans.

The source of mares' serum gonadotrophin has not been definitely established, but the tissue most likely concerned is the endometrium, although the pituitary gland and the chorion cannot be entirely ruled out. Catchpole & Lyons [1934] observed that the hormonal activity of the endometrium of the pregnant horn of the uterus greatly exceeds that of corresponding tissue from the non-pregnant horn. Since they considered it unlikely that the hormone, if secreted by the pituitary gland, would be absorbed selectively by the pregnant horn, they concluded that the hormone is secreted by the chorion and that it passes through the endometrium into the maternal blood stream.

The gonadotrophic activity of the oval cup-like structures that protrude from the surface of the pregnant mare's endometrium was shown by Cole & Goss [1943] to be as great as 8000 i.u./g. of fresh tissue. With one exception, these observations are in very close agreement with those of a corresponding study which Dr Rowlands had recently carried out.

The endometrial cups are arranged in a horseshoe-shaped manner along the outer curvature of the uterus with the two ends of the horseshoe pointing towards the intercornual septum. Many of the larger cups, measuring up to 5 cm. in length and about 2 cm. in width, contain a straw-coloured viscous gel which is adherent to the chorion. Cole & Goss observed that the potency of this substance, collected from three mares, averaged no less than 170,000 i.u./g. of fresh material. The maximum figure obtained by Dr Rowlands was, however, only about 14,000 i.u./g.

The endometrial tissue of the pregnant horn contained only about 1/25th the amount of gonadotrophin found in the cup tissue. The potency of the endometrium of the non-pregnant horn was only 30-70 i.u./g., and was similar to that of other body tissues, representing the activity probably imparted to them by residual blood.

The endometrial cup consists of a mass of extremely active secretory cells which, along the outer surface of the cup, are undergoing symplasmic degeneration and merge into the gel. Penetrating this mass of cells are the necks of the endometrial glands,

\* Dr G. Brownlee, of the Wellcome Physiological Research Laboratories, assisted in the biological assay of the tissues of the pregnant mares. The histological examination of the endometrial cups was carried out in collaboration with Prof. E. C. Amoroso of the Royal Veterinary College.

which pour a copious secretion into the space between the cup and the chorion. It seems likely, therefore, that the viscous gel which is found in the hollow of the cup consists of a mixture of this secretion and of the degenerating cells of the secretory stromal tissue. These cells, especially those in the lower region of the cup, are in close relation with lymphoid tissue and with small lymph channels which probably connect with the large lymphatic sinuses that are found in profusion beneath the cup.

It seems reasonable to regard these tissues as the probable source of the gonadotrophins in the blood of the mare, but it has not yet been shown whether it is the endometrial or the highly active stromal tissue that is involved in hormone production. If the endometrial glands are responsible, it is difficult to see how the hormone, which would be secreted into the lumen of the uterus; re-enters the maternal circulation. There are no specialized areas of absorption in the vicinity of the cup, and the fact that the hormonal content of foetal tissue is negligible makes it unlikely that the hormone is absorbed by the chorion to enter the foetal circulation. If, on the other hand, the hormone is secreted by the stromal tissue, the presence of lymphatic vessels at the base of the cup indicates its probable route of entry into the maternal circulation. On this view the degenerating stromal tissue would be responsible for the high hormonal content of the cup-gel, and the secretions of the endometrial glands for its bulk.

*Biological activities in recipient species.* There is general agreement that the biological activities of chorionic and mares'-serum gonadotrophins differ fundamentally from each other, the differences being related to their different qualitative make-up and to their differential rates of destruction and excretion in test animals [Hamburger & Pedersen-Bjergaard, 1938].

The differences between the ovarian response to mares'-serum and chorionic gonadotrophins are most evident in the hypophysectomized rat. The response to chorionic gonadotrophin takes the form of intense hyperaemia and hyperplasia of the stromal tissue. Ovarian follicles remain unstimulated. Mares'-serum gonadotrophin stimulates follicular development as a quantitative response depending upon the amount of hormone administered. Ovulation and the formation of corpora lutea occur only very rarely, although luteinization of the membrana granulosa of some of the follicles has been observed. Williams [1945*a, b*], however, has shown that a quantitatively greater response is produced in rats either when serum gonadotrophin is injected immediately after hypophysectomy or when post-hypophysectomy atrophic changes in the ovaries were inhibited by oestrogens given at the time of the operation. The incidence of ovulation and the occurrence of luteinization were greater after such preliminary treatment than in experiments in which the atrophic changes were complete at the start of the test period. It is reasonable to suppose, therefore, that, although mares'-serum gonadotrophin is predominantly follicle-stimulating, it is capable of producing ovulation and luteinization under certain experimental conditions. Chorionic gonadotrophin, on the other hand, only contains the luteinizing factor.

The whole cycle of changes involving follicle stimulation, ovulation and corpus luteum formation cannot be accomplished with any certainty, using either of these hormones independently. With correct dosage and timing, however, mares'-serum gonadotrophin followed by chorionic gonadotrophin can induce all stages of the cycle with great regularity [Rowlands & Williams, 1943].

The optimal interval between the two injections in hypophysectomized rats was found to be 4 days, which is presumably the time taken for the follicles to grow from their atrophic condition to full maturity under the influence of the serum gonadotrophin. In a parallel set of investigations in the intact rat [Rowlands, 1944], it was found that the optimal interval between the two injections was 2-3 days, and that the incidence of ovulation fell as the interval was extended. The time taken by mares'-serum gonadotrophin to induce follicular maturation in the intact immature animal, therefore, corresponds to the duration of the follicular phase in the oestrous cycle of the adult rat. It is conceivable that for the most successful clinical use of serum gonadotrophin, the rate of follicular stimulation should approximate that which is natural for the normal adult of the species.

Ovulation, in both normal and hypophysectomized rats previously treated with serum gonadotrophin, occurred within 12-13 hr. after the injection of chorionic gonadotrophin, and ova began to appear in the Fallopian tubes 1 hr. later.

Subsequently Rowlands & Williams [1946] were able to show that hypophysectomized rats treated in this way became sexually receptive and that, when mating occurred, no less than 60 % of the ova recovered from the uterine tubes 2 days later had undergone normal blastomeric segmentation. The proportion of degenerating eggs, however, was considerably greater than normal. Observations on the ultimate fate of these fertilized eggs were not made.

*Action and function of the anterior-pituitary-like (A.P.L.) hormones in donor species.* It is unlikely that the A.P.L. hormones are required by the foetus because only small proportions can be detected in the foetal circulation. In the foetus of the mare, this amount is, nevertheless, capable of causing a temporary enlargement of the gonads. No detailed histological data on the human (maternal) ovary during early pregnancy are available, but the literature contains frequent references to the occurrence of extensive luteinization and to the formation of luteal cysts in the ovaries of fetuses when a chorion epithelioma is present in the maternal organism, and when there is much chorionic gonadotrophin in the urine [Zondek, 1942]. Furthermore, Hisaw [1944] has shown that when chorionic gonadotrophin is injected into monkeys during the luteal phase of the cycle, the onset of the ensuing menstrual bleeding is very considerably delayed. This treatment, he considers, maintains a functional corpus luteum for an additional 10-15 days. Evidence of this kind favours the view that chorionic gonadotrophin is secreted during early pregnancy and has the effect of prolonging the life of the corpus luteum, and of ensuring an adequate level of progesterone in the blood during the transition period when the placenta becomes established as an endocrine organ, and takes over the function of the declining corpus luteum.

While gonadotrophin is present in abundance in the blood, the ovaries of the pregnant mare contain follicles up to about 3 cm. in diameter, as well as corpora lutea of different ages, ranging from slightly luteinized follicles to old fibrotic luteal masses. The luteal tissue is diffuse, and often the primary corpus luteum of pregnancy cannot be distinguished. According to Kimura & Lyons [1937], the latter regresses at about the end of the first month and, subsequently, new corpora lutea continue to appear in both ovaries until about the end of the fourth month. It is doubtful whether ovulation occurs; the corpora lutea appear to arise from luteinization of the theca of

the follicles. Nevertheless, their progesterone content is considerable. As implantation of the blastocyst does not occur until about the 40th day [Ewart, 1915], the secondary corpora lutea probably help to maintain a pregestational endometrium when the primary corpus luteum is in decline.

If, then, the function of the A.P.L. gonadotrophins is the formation of secondary corpora lutea and of lutein tissue in the maternal ovaries, it would seem that the method by which this tissue is formed varies between different species. It is unlikely that the degree of follicle stimulation which occurs in the human ovary during early pregnancy is anything as great as that which occurs in the mare. It is also surprising that the level of blood oestrogen in the mare does not rise until after the disappearance of the gonadotrophin from the blood, rather than during the period when the ovaries contain many follicles [Cole & Saunders, 1935].

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In commenting on Dr Rowland's paper, Dr BACSICH pointed out that it is generally recognized that the source of the chorionic gonadotrophins is the trophoblast. He emphasized the disappearance of the cyto-trophoblast in the human placenta around the end of the fourth month, and asked whether the reduction of the placental barrier is simply a means of improving placental efficiency, or if the reduced excretion of chorionic gonadotrophins after the first 60-90 days of pregnancy could also be connected with the gradual ageing and disappearance of the cyto-trophoblast.

His own studies, carried out with Dr Wyburn, were in agreement with those of Dr Rowlands, as they had observed only extensive follicle stimulation and some luteinization, but no ovulation, in immature female guinea-pigs after the administration of PMS gonadotrophins (Antostab).

There are numerous multinuclear ova and multiovular follicles in human infant ovaries during the first months of life. He believed that these abnormalities are correlated with the extensive rate of atresia of primordial ova which takes place after birth, and that they are due to the sudden postnatal withdrawal of the placental gonadotrophins. It is generally held that the gonadotrophins have no effect on the foetal gonads, but on the basis of the above observations, he felt that it is justifiable to assume that the histological differentiation of the gonads during foetal life is governed in some way by gonadotrophins.

**Gonadotrophins and sex hormones in cases of malignant tumours of the testis.\*** By Dr C. HAMBURGER. *State Serum Institute, Copenhagen, Denmark.*

Certain malignant tumours of the testis, e.g. chorion epitheliomas, and related tumours classed as mixed epitheliomas, which characteristically contain syncytial trophoblast-like cells, produce chorionic gonadotrophin. Other testicular tumours, e.g. seminomas, are associated with the excretion of a hormone that is identical in its biological properties with the follicle-stimulating principle elaborated by the anterior lobe of the hypophysis. Because mixed epitheliomas are extremely malignant, and are also resistant to X-ray treatment, while seminomas are sensitive to X-rays and more benign, gonadotrophin analyses in patients suffering from testicular growths have a considerable diagnostic and prognostic value. Between 300 and 400 such cases have been submitted to hormone analysis in Denmark.

Biological tests on hypophysectomized rats, on chickens, pigeons and monkeys, together with comparisons of dose-response curves for ovarian and uterine weights in normal immature rats, make it possible to differentiate clearly between chorionic gonadotrophin, pregnant-mare-serum gonadotrophin, and hypophysial gonadotrophin. A clear distinction between chorionic gonadotrophin and follicle-stimulating hormone from the hypophysis is also provided by the different histological changes which these substances induce in the ovaries of immature mice.

Chorionic gonadotrophin is demonstrable in large quantities in extracts of chorion epitheliomas and related tumours. The excretion of this hormone is roughly proportional to the amount of tumour tissue in the body. Since chorionic gonadotrophin cannot be demonstrated in the pars glandularis of patients who excrete the hormone, it is therefore reasonable to suppose that it is actually elaborated by the tumour tissue. The F.S.H. excreted by the majority of patients with seminomas is identical, in biological properties, with that found in the urine of castrates or of post-climacteric women. Excretion of this substance does not cease after the removal of the tumours, and bears no relation to the presence or absence of metastases. Furthermore, it has never been demonstrated either in the primary tumour or in the metastases of seminoma tissue. The inference, therefore, is that the F.S.H. found in patients suffering from this type of tumour is secreted by the hypophysis.

The androgen excretion of such patients is usually as low as that of castrated men. It is reasonable to suppose, therefore, that the F.S.H. excreted by these patients is a secondary effect of the low androgen production, as it is in castrates and in hypogenitalism. Why the patients suffering from seminomas should produce so little androgen is not immediately clear. Factors which may be responsible are (1) the destruction of testicular tissue by the tumour, and (2) the toxic effects of the tumour, associated with the effects of repeated X-ray treatment.

A review of the general evidence provided by the study of 400 instances of malignant testicular tumours, and of the special evidence provided by the detailed analysis of certain cases, leads to the following conclusions:

(1) Some testicular tumours and their metastases, which contain cells related to chorionic epithelium, produce chorionic gonadotrophin and, in certain cases, oestrogen.

\* Work carried out in collaboration with Dr F. Bang of the Radium Station, Copenhagen, and Dr J. Nielsen.

(2) Other testicular tumours, including seminomas, lead to the increased production and excretion of hypophysial follicle-stimulating hormone. This effect is a secondary one due to a decreased production of androgenic hormone.

(3) In rare cases, follicle-stimulating hormone and chorionic gonadotrophin are excreted successively, and even more rarely simultaneously, by the same patients.

**Oestrogen level and ovarian hypophysial relationship during pseudo-pregnancy and pregnancy in the rabbit.** By Dr MARC KLEIN. *University of Strasbourg, France*

Since the pioneer work of Beard and Prenant, it has been generally accepted that the main functions of the corpus luteum are to inhibit follicular ripening and to prevent oestrus. Hammond [1925], in his monograph on the rabbit, concluded that 'the prevention of ovulation by the corpus luteum was due, not to its internal secretion, but 'rather to both corpus luteum and follicle being dependent on the same substance for their development and that the active corpus luteum has a priority call on this substance so that complete maturity of the follicle is prevented'. In subsequent experimental work it has been generally assumed that this 'same substance' is a hormone of the anterior pituitary.

It is possible, however, that the 'substance' is an oestrogen. Numerous observations support this view, especially the experimental data showing that the functional activity of the corpus luteum is under the close control of oestrogen.

Klein & Mayer [1942] recently tried to affect the corpus luteum by destroying the middle- and large-sized follicles in rabbits' ovaries, and were surprised to find that new follicles had ripened in an ovary where flourishing corpora lutea remained. As a result it was clearly impossible to maintain the strict view of an inhibitory action of the corpus luteum on follicular ripening. Later they [Klein & Mayer, 1945] studied the behaviour of female rabbits at the end of pseudo-pregnancy and pregnancy, and found that such animals, if treated with oestrogen, would mate when normally they do not. The action of oestrogens was, therefore, investigated during all stages of pseudo-pregnancy and pregnancy, when flourishing corpora lutea are always present in the ovaries, and when the females are normally anoestrous in behaviour [Klein & Mayer, 1946]. The following technique was used, first with pseudo-pregnant and later with pregnant rabbits.

Rabbits in oestrus were mated with a vasectomized buck. During pseudo-pregnancy, from the sixth to the eleventh day after mating, 200-300 i.u. of oestradiol benzoate were injected daily. The day after the last injection, the female was mated a second time with a sterile buck. In all cases mating took place. Then the rabbits were separated into two groups. In the first, the injections of oestrogen were continued; in the second, the injections were stopped. The animals were killed as a rule 5 days after the second mating. New follicles had ruptured and new corpora lutea developed in both sets of animals. In the one in which the oestrogen treatment was continued, two sets of corpora lutea were flourishing. In the other, the first generation of corpora lutea was breaking down, while the second set had developed normally. The endometrium of the first group showed the syncytial development that occurs during normal pregnancy, or when oestrogens are injected at the end of pseudo-pregnancy. In the

second group, a new endometrial reaction had occurred, and corresponded in appearance approximately to the age of the second generation of corpora lutea.

In short, when rabbits at different stages of pseudo-pregnancy are injected daily with 200 i.u. oestrogen, a second mating takes place, despite the presence of active corpora lutea. A set of follicles bursts and gives rise to a new generation of corpora lutea. A second pseudo-pregnancy begins which interferes with the first one. The whole process starts with the injection of oestrogen, and does not require the injection of gonadotrophin.

Similar experiments were carried out on normal pregnant rabbits. Mating with the sterile vasectomized buck took place successfully. Despite pregnancy and the presence of active corpora lutea in the ovaries, follicles ruptured and new corpora lutea appeared. The new pseudo-pregnancy had variable effects on the progress of the established pregnancy, but these will not be reported here. The main fact to be noted is the occurrence of a second mating during pregnancy as a result of previous oestrogen treatment and without injection of gonadotrophin.

In a further series of experiments, pregnant rabbits were mated with normal bucks, after the following preliminary treatment. On the eighth day of pregnancy, just before the normal time of insertion of the blastocysts, laparotomy was performed and one of the uterine horns was emptied. Three days later the animals were injected daily with 150 i.u. of oestrogen, and after a few days were mated again with a normal male. Nine days after this second mating, the animals were killed. The results varied somewhat, but typically the ovaries contained two sets of corpora lutea. The older one had greatly regressed, the new one was fully developed. Degenerating remnants of the first pregnancy were present in the unoperated uterine horn, while the emptied horn contained the normal products of the second mating. These results show that the injection of oestrogen alone will induce pregnant rabbits to mate a second time with a normal male, and that this mating is followed by rupture of follicles, new corpora lutea, fertilization of the eggs and normal insertion of the blastocysts.

Despite the presence of functional corpora lutea, therefore, the neurohormonal reflex following mating will result in the rupture of follicles and the development of new functional corpora lutea. Oestrous behaviour, like anoestrous behaviour, seems to be a matter of oestrogen level. This level is normally lowered during the period of luteal activity, but, nevertheless, oestrous behaviour can be restimulated by raising the oestrogen level experimentally.

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Dr MALPRESS, discussing Dr Klein's experiments, suggested that they afforded additional evidence for the existence of a luteotrophic hormone. Stimulation of the release of this hormone by oestrogen might explain the perpetuation of corpora lutea in Dr Klein's oestrogen-treated pseudo-pregnant animals. This interpretation would accord with the view that luteotrophin is identical with prolactin, whose release is believed to be stimulated by low concentrations of oestrogen.

In further discussion, Dr FOLLEY inquired how Dr Robson would explain Lyons's experiments in which pregnancy was maintained by prolactin in rats hypophysectomized after mating. The prolactin was said to be a pure protein hormone, and uncontaminated with gonadotrophin; it would, therefore, seem that these experiments are further evidence of the luteotrophic action of prolactin. In Dr Folley's view prolactin should be regarded as a third gonadotrophin capable of maintaining the function of existing corpora lutea.

Dr ROBSON suggested that it would be useful to repeat the Lyons experiments on rabbits, and that in spite of what had been said, there was clear evidence for a direct effect of oestrogen on the corpus luteum.

In further comment, Dr Klein remarked that it was possible that oestrogen acted upon the corpora lutea both directly and indirectly through the pars glandularis.

### **The mitogenic action of the sex hormones. By Dr W. S. BULLOUGH.**

*Department of Zoology, University of Sheffield*

Recent work has suggested that cycles of mitotic activity occur in the various tissues of the adult female mouse throughout the oestrous cycle. In such tissues as those lining the uterus, vagina, duodenum and rectum, mitotic activity reaches a peak just before the end of dioestrus, is low again throughout early oestrus, and then rises again to a second peak at full oestrus. In the epidermis the first peak does not occur before pro-oestrus, after which there is only time for a second incomplete peak in late oestrus. In the pancreas and the linings of the ureter and urethra, on the other hand, only one peak is observed at full oestrus. It is clear that the body tissues vary widely in their sensitivity to oestrogen. This conclusion is supported by the results of oestrone injection experiments. A second conclusion is that once high mitotic activity is achieved in any tissue, it is immediately suppressed, in spite of the continued presence of high concentrations of oestrogen. Some mechanism termed the mitosis depressor is postulated to explain this effect. The action of the depressor continues for about 24 hr. when, if the oestrogen concentration is still sufficiently high, a second peak of activity follows.

The ovarian cells are highly resistant to the mitosis stimulating effect of oestrogens, and mitotic activity in the ovary is limited to the immediate vicinity of the pools of liquor folliculi. Secretion of this liquor, rich in oestrogenic hormone, and mitotic activity of the follicle wall start about the second day of dioestrus, and result in the most rapid follicle growth. Mitotic activity is greatest in those cells of the membrana granulosa which are in actual contact with the liquor folliculi, and lowest in the theca externa. As in other body tissues, once mitotic activity has reached a high level it dies rapidly away so that during oestrus, follicle growth is almost entirely due to an increase in the volume of the liquor folliculi. As a consequence, the wall becomes thinner and thinner until it finally bursts. A second but much smaller wave of cell division precedes the luteinization of the corpus luteum, and again the distribution of the mitoses bears a marked relation to the disposition of the liquor folliculi.

In the germinal epithelium, mitoses are first evident in those cells overlying the rapidly growing follicles. However, maximum mitotic activity is not induced until



after ovulation, when the epithelium is actually bathed by the extruded liquor folliculi. As many as 200 mitoses may then be counted round each ruptured follicle, and this effect can also be readily induced by bathing the ovary with injected oestrone, preferably in oil solution.

It would seem that the primary action of the oestrogenic, as also of the androgenic, hormones is to induce mitosis in the various body tissues. Their effect is greatest in those epithelial tissues which have the greatest cell wastage, and it is interesting that they occur widely in both animal and plant kingdoms. It appears possible that they have been evolved as sex hormones only in the subphylum Vertebrata.

Dr KORENCHEVSKY, in commenting on this paper, presented a summary of certain experiments which suggested that thyroid extract was very much more mitogenic than oestrogen. The combination of the two substances produced an even more marked effect on the mitotic activity and growth rate of a variety of tissues. In his view conclusions such as those stated by Dr Bullough required a basis in tissue-culture experiments, and not in experiments in which a number of other bodily factors operated independently of the experimenter's control. Dr Bullough's comment on this point was that the action of thyroid was probably general, since it increased the basal metabolic rate, and through this the rate of cell division.

Prof. ZUCKERMAN referred to the fact that certain of Dr Bullough's general conclusions conflicted with observations on the effects of oestrogenic stimulation in other species. For example, proliferation of the cellular elements of the monkey's endometrium appeared to continue uninterruptedly, however prolonged the phase of oestrogenic stimulation, and there was no suppression of this activity as suggested by Dr Bullough. There was also a gradient of sensitivity in the skin, the circumgenital area of the monkey being the most responsive to oestrogen. Prof. Zuckerman also remarked on the fact that Dr Bullough's experiments did not refer to oestrogenic stimulation alone, but to the effects of treatment with colchicine as well.

Dr Bullough replied that the results obtained for the mouse epidermis were from analyses made on the antero-dorsal and postero-dorsal regions of the body, and on the ears. They did not refer to any area close to the genital region.

### Gonadotrophic effects in farm animals. By A. L. GREENBAUM.

*National Institute for Research in Dairying, Shinfield, Reading*

Among the pioneer studies on the effect of gonadotrophins on the larger domestic animals, those of Cole and Miller on sheep were of outstanding importance. Hammond & Parkes underlined the practical implications of this work when they pointed out that PMS might be of considerable value in increasing the fertility of normal matings in sheep, or in making breeding possible during the non-breeding season of those animals which have failed to lamb at the normal time. One of the disadvantages in the use of PMS for this purpose is the fact that although single injections usually cause ovulation they only occasionally induce oestrus. Two injections, spaced 16 days apart (16 days is the normal length of the oestrous cycle in sheep during the breeding season), produce both ovulation and heat, and in some cases initiate cyclic behaviour

during what is normally the period of anoestrus. Much work along these lines has been done in the U.S.S.R.

Experiments on cows have not been on an ambitious scale, although their economic importance in Great Britain calls for an intensive study of the application of gonadotrophic hormones to the production of twins in beef cattle, and the problem of bovine sterility in dairy cattle. In spite of their superficial resemblance, these two problems are not identical. In dairy cows treatment must not only induce ovulation but must also ovulate only one follicle at a time, since the female offspring of a mixed pair of calves is often a freemartin. In the case of beef cattle, freemartins are said to have superior fattening qualities, and their production might even be an advantage.

Folley and Malpress treated four groups of heifers with single subcutaneous injections of 1000, 2000, 3000 and 4000 i.u. of PMS. The animals were killed at intervals of up to 14 days after the date of injection. A further group of control uninjected animals was examined at the same time, and in these only one animal was found with an ovarian follicle greater than 15 mm. in diameter. Among the injected animals several changes in ovarian structure were apparent, the most clear-cut being an increase in mean follicular diameter and in the percentage of follicles with a mean diameter greater than 10 mm. Fourteen days after 4000 i.u. PMS the mean follicular diameter had risen to 20 mm., that is, three times the control value, with some follicles greater than 30 mm. Hammond has pointed out that the normal size of the bovine follicle at ovulation is about 15 mm. Thus, when the majority of follicles reached ovulation size, the conditions for ovulation cannot have obtained, with the result that the follicles continued to grow.

The ovaries also increase in weight, but the individual variations after the fifth day are very considerable. In view of the current practice of measuring gonadotrophic activity in small animals by the increase in ovarian weight, it is of interest to note that, in the bovine, changes in ovarian weight are much less reliable indicators of gonadotrophic activity than either the mean follicular diameter or the percentage of large follicles.

A study of the long-term effects in cows injected with 3000–4000 units and killed at intervals up to a year after injection, revealed that in general the ovaries returned to normal with no permanent impairment of breeding potentiality.

Some of the animals used in this work were in anoestrus while others showed normal cyclic behaviour. The state of the ovary at the time of the injection had therefore to be considered. Experiment showed that the presence of an active corpus luteum has very little inhibitory effect on the action of PMS on follicular growth, but that it does have a profound effect on ovulation. Of fifty-seven animals injected with doses of 3000 or 4000 units in the first 14 days of the cycle, that is, during the phase of luteal activity, only three ovulated, none of these being multiple ovulations. Whereas of seventeen animals injected in the follicular phase of the cycle, that is, in the last 7 days, fourteen ovulated, eight of these releasing more than two ova.

Quantitatively the ovarian changes induced in the cow by horse-pituitary extracts were, in general, similar to those evoked by PMS. They differed, however, in that the growth of follicles after injection was linear for only about 10 days, when they measured about 12 mm., thereafter falling fairly rapidly, whereas the follicles in the

ovaries of cows treated with PMS were still growing actively after 14 days, when they were 20 mm. in diameter.

The data on ovulation after the two treatments show several points of interest. Horse-pituitary extracts occasionally induced ovulation within 1 or 2 days after injection, and before any dimensional follicular changes could have taken place. This effect is presumed to be due to the shock of the sudden impact of exogenous gonadotrophins, probably mainly the luteinizing fraction, on a medium-sized follicle in the ovary, causing premature rupture.

With a single exception ovulation was never caused by injection of PMS, except when given during the follicular phase of the cycle. Horse-pituitary extracts, however, caused ovulation at any stage of the cycle, the decisive factor being, apparently, the presence in the ovaries of one or more follicles sufficiently large to respond, rather than the absence of a corpus luteum.

The failure of PMS to cause ovulation except during the follicular phase of the cycle is explicable on the current thesis that the oestrogen of the ovary triggers the release of the luteinizing hormone of the pituitary, which causes ovulation. Folley and Malpress have shown that the membrana granulosa of follicles, which probably secretes the oestrogen, is severely attenuated in PMS-stimulated follicles. Thus in spite of the considerable increase in the area of this layer, the amount of oestrogen it secretes may not reach the threshold necessary to stimulate the pituitary. Some support for this supposition is provided by the fact that in spite of the huge follicles in the ovaries of treated animals, the cows do not become nymphomaniac, as they probably would with a cystic follicle of comparable size. When injections are given in the follicular phase, fair-sized follicles, with granulosa layers in an advanced stage of development, are already present. They do not have to suffer the thinning out process to reach ovulation size, and the mechanism for release of the pituitary hormone can proceed in the normal manner.

Although the results indicated above were obtained for the most part on cyclic heifers, and some reinterpretation is probably necessary before they can be applied to the hypoplastic ovaries of the sterile cow, it does seem to be true that neither PMS nor pituitary gonadotrophins are of much use in the treatment of this condition in dairy cattle, since it appears impossible, at the moment, to guarantee the ovulation of only one follicle.

The anoestrous goat does not respond to these treatments in the same way as sheep. Thus, although a single injection of PMS readily induces ovulation in the majority of sheep, comparatively few show signs of heat, and unless artificial insemination is employed the ova are wasted. The goat, on the other hand, not only ovulates after a single injection, but usually shows symptoms of heat as well.

As in the cow, the increase in mean follicular diameter is a more reliable measure of gonadotrophic activity in the goat than increase in ovarian weight. No significant change in diameter occurs with doses up to 400 units, but doses above this level begin to take effect.

The most useful index of gonadotrophic activity was found to be the proportion of follicles greater than 6 mm. The value of the index increases with dose, and, conversely, the percentage of follicles less than 1 mm. progressively falls. The threshold value for follicular stimulation was found to be about 600 units. On a weight for weight basis

this corresponds to about 6000 units for the cow—which is considerably less than the figure of 1500–2000 units found by Folley and Malpress, and by Hammond Jnr. and Battacharya. Provided the dose is above 600 units, most of the goats treated come into season after a single injection. At least one ovulation occurred in each group injected with dosages up to 600 units. The mean number of ovulations was roughly constant for higher doses, and somewhat above the normal physiological figure, which in the goat is usually 1 or 2 and exceptionally 3. Washing the uterine tubes resulted in a recovery of some 60 % of the eggs. Out of more than 100 eggs recovered only three were found to be dividing.

The experiments do not throw much light on whether the eggs produced in goats as a result of experimental gonadotrophic stimulation are normal, or whether the genital tract has been suitably prepared for the passage of sperm and the implantation of the fertilized ovum. Very few of the ova shed as a result of treatment with gonadotrophins became fertilized. Our own experiments, and those of Phillips, Frap and Frank, indicate that the failure to secure kids in these animals occurs at the stage of fertilization, but the problem requires further investigation.

In summing up the symposium, Dr BISHOP remarked that it had showed that we were still far from a clear understanding of three basic problems: first, that of the ways by which the gonadotrophic functions of the pituitary gland are controlled by environmental changes; secondly, that of the manner of the interactions of gonadal and gonadotrophic hormone effects; and thirdly, that of the number and source of specific gonadotrophins produced by the tissues of the body. Much research remained to be done before these fundamental questions were elucidated.

## ORDINARY MEETING

*Held at the Zoological Society, London, on 29 January 1947*

**A closed vessel technique for the assay of thyroïdal activity.** By A. U. SMITH, C. W. EMMENS and A. S. PARKES. *National Institute for Medical Research, Hampstead, London, N.W. 3*

Raising the temperature shortened, and lowering it prolonged the survival time of mice in closed vessels. A correlation existed between survival time and body weight, but was unimportant in the range 16–24 g. Survival time increased in proportion to the volume of the vessel. Treatment with iodinated casein shortened the survival time, and dose-response curves, constructed by plotting dose on a logarithmic scale against the mean survival time of groups of ten mice, were linear. The effect was maximal on the second day and progressively less on the first, fourth, and eighth days after the last injection. Survival times of mice treated for 1, 2 and 3 weeks with iodinated casein were not significantly different. Absorption of CO<sub>2</sub> in the jars by soda lime prolonged survival times, both of iodinated casein-treated and control mice.

A factorial test showed that the dose-response curve was steeper in 2 lb. than in 1 lb. Kilner jars, but that the use of soda lime was of no advantage. Male mice were less variable in their response to iodinated casein than female mice.

Using the survival time in 2 lb. Kilner jars at 23° C. of male mice weighing 16–20 g. an assay with forty mice per substance should give a result within 80–124 % of the true values in nineteen out of every twenty tests made.

**A simple mammalian test for thyroidal activity.** By A. U. SMITH, C. W. EMMENS and A. S. PARKES. *National Institute for Medical Research, Hampstead, London, N.W.3*

Experiments were carried out on the survival time of mice in sealed vessels of different size. Survival time in 2 lb. Kilner fruit bottling jars was 2–3 hr.—a suitable period for experiment. A preliminary experiment on survival time of female mice receiving treatment with different doses of thyroid, as compared with that of casein-injected controls, showed that there was a close linear relation between dose and survival time.

The next step was to investigate the composition of the residual air in the bottles at 30 min. intervals after the start of the experiment or up to the death of the animals. These results were rather unexpected in that the decrease in the oxygen content, and the increase in the carbon dioxide content, was no more rapid when the mice had had preliminary treatment with thyroid. The point at which death occurred, however, was entirely different. Thus, on the average for normal mice, death took place when the oxygen was down to about 4 %, and the carbon dioxide up to about 14 %, while in thyroid-injected mice death occurred when the oxygen was still above 8 % and the carbon dioxide content had just reached 10 %. We must conclude, therefore, that when the mice had received preliminary treatment with thyroid over a period of one week their decreased survival time in sealed vessels was due to reduced resistance to anoxia rather than to increased consumption of oxygen.

It appears, therefore, that the survival time of mice in sealed vessels provides an easy, inexpensive, and rapid mammalian method for the estimation of thyroidal activity. Such a method is much needed for the assay of iodinated proteins for which the estimation of acid-insoluble iodine does not provide a reliable index of biological activity.

**The water-balance principle of crustacean eye-stalks.** By H. HELLER and BARBARA SMITH. *Department of Pharmacology, University of Bristol*

Confirming Gray & Ford (1940) it was found that extracts of crustacean eye-stalks contain a principle which increases the uptake of water by frogs. Neurohypophysial extracts have a similar 'water-balance effect' on amphibians (Heller, 1945). The question whether crustacean eye-stalk extracts show any of the other actions of the mammalian posterior pituitary hormones was therefore investigated. The effect of crustacean eye-stalk extracts on the blood pressure of the spinal cat, on the water diuresis of the unanaesthetized rabbit, on the virgin guinea-pig uterus and on the urinary chlorine excretion of rats (Dicker & Heller, 1946) was examined. No

significant response was obtained in any of these tests. There is therefore no evidence to show that the crustacean and the vertebrate water-balance factors are homologous principles.

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#### **Halogen-containing oestrogens.** By C. W. EMMENS. *National Institute for Medical Research, Hampstead, London, N.W. 3*

A full report of this paper has been submitted for publication in the *Journal*.

#### **The effect of propyl-thiouracil on the oestrous cycle in mice.** By P. L. KROHN. *Department of Anatomy, University of Birmingham*

The normal oestrous rhythm of mature albino mice was disturbed by the daily subcutaneous injection of 0.3 mg. propyl-thiouracil. The cycles were either lengthened and irregular or disappeared completely. The oestrous rhythm returned again about a fortnight after cessation of the injections.

A single injection of 5 i.u. chorionic gonadotrophin into six mice showing prolonged anoestrus during thiouracil treatment was followed in each case by a single oestrous period.

The vaginal sensitivity of spayed mice to oestrogen was unaffected by propyl thiouracil treatment, and it was suggested that the anoestrus condition may have been due to a diminution in the gonadotrophin secretion of the pituitary owing to increased demands for the production of thyrotrophic hormone.

#### **Effect of sodium intake on adrenalectomized rats receiving cortical steroids.**

By A. T. COWIE, S. J. FOLLEY, T. H. FRENCH and A. L. GREENBAUM. *National Institute for Research in Dairying, Shinfield, Reading*

Gaunt, Eversole & Kendall (1942) reported that 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone were more effective than deoxycorticosterone acetate (doca) in maintaining lactation in adrenalectomized rats, and concluded that the critical factor for normal lactation was a sufficiency of the 11-oxygenated cortical steroids which are concerned with protein and carbohydrate metabolism. Folley & Cowie (1944) were unable to confirm these observations. They found that doca, although not completely restoring lactation, was superior to either of the above 11-oxygenated steroids. The value of doca in replacement therapy was confirmed in further experiments (Cowie & Folley, 1947b). An experiment was then carried out to find whether it was possible to enhance the replacement value of the 11-oxygenated steroids by increasing the protein in the diet to 50 %. This experiment clearly showed,

however, that the lactation response of adrenalectomized rats to the 11-oxygenate steroids was, if anything, reduced by feeding the animals a high-protein diet (Cowie & Folley, 1947c). Kendall (1946) then suggested that the intake of sodium and potassium might have a secondary effect on the action of the 11-oxygenated steroids on gluconeogenesis. It was therefore decided to study the effect of increasing the sodium intake on the lactational performances and responses of adrenalectomized rats.

The following treatments were investigated: (a) controls (rats subjected to sham operation); (b) adrenalectomy (4th day of lactation); (c) adrenalectomy + 0.56 mg. dehydrocorticosterone acetate; (d) adrenalectomy + 0.56 mg. doca; (e) adrenalectomy + 3.0 mg. doca. There were twelve rats on each treatment, six of which were fed on the stock diet (containing 0.33 % Na, 0.88 % K) and six on a high sodium diet (0.67 % Na, 0.88 % K). The number of young per litter was standardized as previously described (Folley & Cowie, 1944; Cowie & Folley, 1947b, c). The steroids were dissolved in sesame oil and the stated dose injected subcutaneously daily. The results confirmed our previous conclusions on the value of doca in replacement therapy in adrenalectomized lactating rats. The responses to treatment (for method of calculation see Cowie & Folley, 1947a, b) were as follows: 3.0 mg. doca gave a 58 % response; 0.56 mg. doca gave a 33 % response; and 0.56 mg. dehydrocorticosterone gave a 13 % response. The lactational performances of the groups on the high sodium diet were in no case significantly superior to the corresponding ones on the stock diet.

As a second criterion of the efficacy of replacement by adrenal cortical hormones after adrenalectomy, liver and mammary gland arginase levels were studied. Fraenkel-Conrat, Simpson & Evans (1943) have shown that adrenalectomy lowers the liver arginase, and Folley & Greenbaum (1946) reported that this reduced liver arginase, and a similar reduction of mammary gland arginase, could be partially restored by adrenal cortical hormones. Using the level of arginase activity as a measure of replacement therapy the efficiency of replacement showed a close parallelism with the results obtained in the lactation experiments, doca being more efficient than 11-dehydrocorticosterone. The results now given show that this effect is not influenced by a high sodium diet, doca remaining far more effective than 11-dehydrocorticosterone acetate in restoring the depleted arginase levels of both mammary gland and liver arginase.

Finally, the liver glycogen levels of our rats, estimated without previous fasting, were found to be depressed by adrenalectomy and to be largely restored by doca, but not by 11-dehydrocorticosterone acetate on either diet.

These results are somewhat surprising in view of the currently accepted theory that it is the 11-oxygenated steroids which are most closely connected with carbohydrate metabolism.

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**Lactogenic hormone and fat metabolism.** By MAX REISS.

*Burden Neurological Institute, Bristol*

The fat content of peripheral fat deposits is reduced after treatment with lactogenic hormone. This was shown on rats where the total fatty-acid content of skins was investigated, and in clinical cases of adiposity, where red striae disappeared shortly after the commencement of the treatment.

Thyroid treatment alone has no effect, but increases the action of the lactogenic hormone.

**Influence of hormones on growth in rats.** By A. ROY. *Department of Biochemistry, University College, Gower Street, London, W.C. 1*

Growth can be induced by the administration of anterior pituitary extract (APE) to (a) thyroidectomized rats, (b) adrenalectomized rats, (c) thyro-adrenalectomized rats and (d) rats previously implanted with diethylstilboestrol (DST). The food intake of the various groups was maintained at the same level as control untreated animals.

Simultaneous administration of APE and thyroxine to normal or thyroidectomized rats produces more growth than the administration of APE alone.

Administration of dinitrophenol has no influence on the growth of thyroidectomized rats.

Administration of APE and adrenocortical extract (ACE) simultaneously to adrenalectomized or normal rats has no more growth promoting activity than the administration of APE alone.

Adrenalectomized rats maintained with 1 % NaCl grow as well as normal rats.

Thyroxine injection has no influence on the growth of normal rats.

ACE injection and the simultaneous administration of ACE and thyroxine inhibit the growth of normal rats. When APE, ACE and thyroxine are given simultaneously, the magnitude of growth is the same as that produced by APE alone.

Short-term experiments lasting two weeks did not reveal the true growth-inhibiting property of DST because both the control and experimental rats lost weight to the same extent. This loss was due to less intake of food. The food intake of experimental animals progressively increases after this phase. Body-weight increases, reaches a weight which is the same as that when the DST pellet was implanted, and then flattens out.

Analysis of the carcass reveals that growth induced in the above animals was due to the deposition of protoplasmic matter.

**The galactopoietic activity of hormones in cows in declining lactation.** By A. ROY. *Department of Biochemistry, University College, Gower Street, London, W.C. 1*

The relative galactopoietic activities of (1) APE, (2) prolactin, (3) thyroxin, (4) adrenocorticotrophin, (5) APE and thyroxin, (6) adreno-corticotrophin and prolactin were studied. All the hormonal preparations were found to be active in stimulating milk production in cows during the declining phase. The responses to combined treatment have been found to be greater than single treatment. The composition of milk obtained during the period of treatment was studied in some of the animals and did not show any significant variation from the normal.





# PROCEEDINGS OF THE SOCIETY FOR ENDOCRINOLOGY

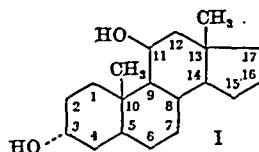
## SYMPOSIUM ON THE ADRENAL CORTEX.

*Held in the Medical School, Birmingham University, on 28 March 1947*

**Recent advances in the chemistry of the adrenal cortex.** By C. W. SHOPPEE.  
*Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W. 3*

A simplified and brief account of the advances made in the chemistry of the adrenal cortex during the last seven years can be dealt with under the four headings of (1) isolation of new substances, (2) constitutional advances, (3) synthetic advances, and (4) connexion between chemical constitution and physiological activity.

For convenience the steroid nuclear skeleton with the appropriate numbering is given in formula (I), which shows an ( $\alpha$ )-orientated substituent hydroxyl group at C<sub>3</sub> (position below the general plane of the ring system being represented by a broken-line bond), and a ( $\beta$ )-orientated substituent hydroxyl group at C<sub>11</sub> (position above the general plane of the ring system being represented by a full-line bond).



### (1) Isolation of new substances

Six new pure crystalline substances have been isolated: oestrone,  $\Delta^4$ -androstene-3:17-dione, 17-( $\alpha$ )-hydroxyprogesterone, an  $\alpha\beta$ -unsaturated ketone of unknown structure (possibly a D-homoandrostane derivative), substance U and substance V. Table 1 gives a complete picture of the present position using Reichstein's alphabetical nomenclature.

Table 1

C <sub>21</sub> O <sub>3</sub> group	C <sub>21</sub> O <sub>4</sub> group		C <sub>21</sub> O <sub>5</sub> group	
J	P	R	A	C
O	K	N	D	E
L	S*	T	F*	M*
Deoxycorticosterone*		Corticosterone*	U	V
17-( $\alpha$ )-Hydroxyprogesterone		11-Dehydrocorticosterone*		

1 compound of unknown constitution with C<sub>21</sub>O<sub>4</sub>; *allopregnan-3( $\beta$ )-ol-20-one* (C<sub>21</sub>), progesterone (C<sub>21</sub>); androstane-3( $\beta$ ):11( $\beta$ )-diol-17-one (C<sub>19</sub>), adrenosterone (C<sub>19</sub>), androst-4-ene-3:17-dione (C<sub>19</sub>); oestrone (C<sub>18</sub>).

\* Physiologically active.

### (2) Constitutional advances

C<sub>3</sub>. By the conversion of substance A into androstane-3-( $\beta$ ):17( $\alpha$ )-diol [Shoppee, 1940] the presence of an oxygen atom at C<sub>3</sub> in substances A, C, D, E, F, M, U and V is proved; it is present as a 3-( $\beta$ )-hydroxyl group in substances A, D and V. By the

conversion of substance A into substance N [Shoppee & Reichstein, 1940], it was shown that the foregoing proof extends to substances R, N, T, corticosterone and 11-dehydrocorticosterone, and that the oxygen atom in question is present as a 3-( $\beta$ )-hydroxyl group in substances R and N.

$C_{4,5}$ -Double bond. Proof that the double bond conjugated to the carbonyl group at  $C_3$  in corticosterone and 11-dehydrocorticosterone must be at  $C_{4,5}$  was achieved by the reduction of the former to a mixture of pregnane-3:17-dione and *allopregnan*-3:17-dione [Shoppee & Reichstein, 1941].

$C_{11}$ . As a result of the proof that the substances of the  $C_{21}O_5$  group possess an oxygen atom at  $C_3$ , it follows by exclusion that these substances, and also those of the  $C_{21}O_4$  subgroup, must possess an oxygen atom at  $C_{11}$  [Steiger & Reichstein, 1937]. In those natural cortex compounds in which the oxygen at  $C_{11}$  is present as a hydroxyl group, proof has been given that this possesses the ( $\beta$ )-orientation [Reich & Reichstein, 1943]. It should be pointed out that owing to the use during 1943-6 of the old incorrect formula for deoxycholic acid, the natural 11-( $\beta$ )-hydroxy-cortex compounds were incorrectly described as 11-( $\alpha$ )-hydroxy-compounds.

$C_{17}$ . Following the proof [Reichstein & Sorkin, 1946] that deoxycholic acid is a 12-( $\alpha$ )-hydroxy compound with the side-chain at  $C_{17}$  ( $\beta$ )-orientated, it is almost completely certain that the 17-hydroxy-cortex compounds are all 17-( $\alpha$ )-hydroxy compounds and not, as previously thought, 17-( $\beta$ )-hydroxy compounds; thus 17-hydroxyprogesterone and the cortical hormones S, M and F are all 17-( $\alpha$ )-hydroxy compounds.

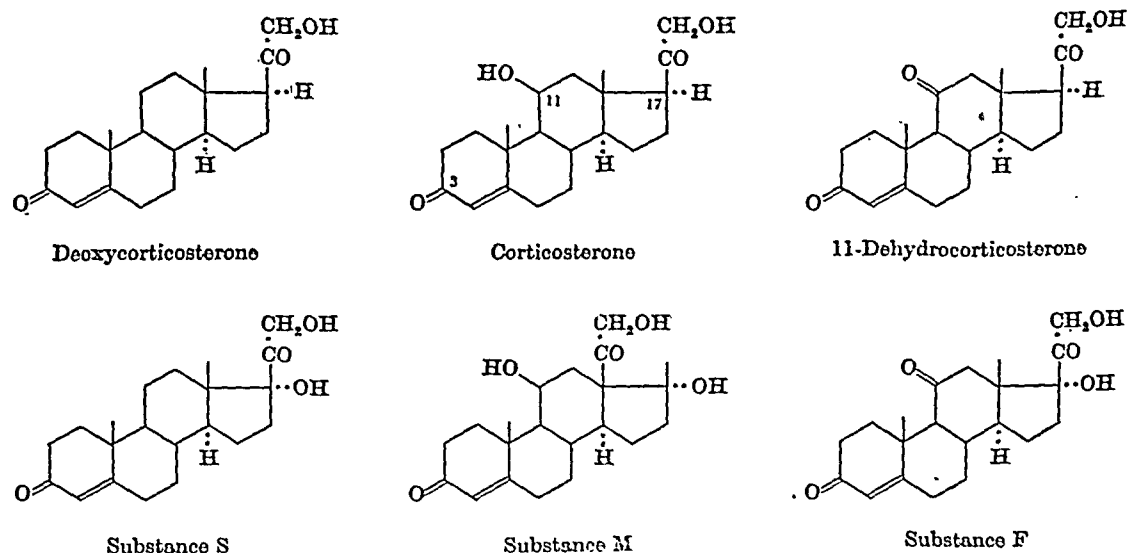


FIG. 1.

### (3) *Synthetic advances*

Only the briefest reference can be made here to Reichstein's syntheses of 11-keto- and 11-( $\beta$ )-hydroxy-steroids, and the subsequent American duplication and variations. All the six cortical hormones shown in Table 1, with the exception of substance M, have now been synthesized from cholesterol.

(4) *Chemical constitution and physiological activity*

The molecular features essential for high activity appear to be (a) the 3-keto- $\Delta^4$ -grouping, (b) the 17-( $\beta$ )-orientated side-chain of the form  $-\text{CO} \cdot \text{CH}_2\text{OH}$ . In regard to (a), the activity in the life maintenance test reported by Selye [Selye, 1941; Waterman, Danby, Gaarenstroom, Spanhoff & Uyldert, 1939] of 3-hydroxy-21-acetoxy- $\Delta^5$ -pregnene-20-one is possibly due to *in vivo* oxidation to the corresponding 3-keto- $\Delta^4$ -compound, since Mamoli [1939, cf. Butenandt, 1942] has shown this to occur *in vitro* with yeast. In regard to (b), it may be noted that replacement of the 21-hydroxyl group in deoxycorticosterone by hydrogen to give progesterone leads to a tenfold decrease in activity and that 17-isodeoxycorticosterone, which differs only in having the side-chain ( $\alpha$ )-orientated, is completely inactive. Provided, however, the side-chain remains ( $\beta$ )-orientated, the 17-hydrogen atom may be replaced by a hydroxyl group without destruction of activity. For activity in regard to carbohydrate metabolism, or, as Long has recently suggested, protein metabolism, an oxygen atom at  $\text{C}_{11}$ , either as a keto-group or as a ( $\beta$ )-orientated hydroxyl group, appears to be essential.

The pure crystalline substances isolated from cortical extracts represent only a small fraction of the activity of the last concentrate; the greater part of the activity (c. 90%) remains in the so-called amorphous fraction, which shows anomalous activities in the various test methods. As will be seen from Table 2, the amorphous

Table 2

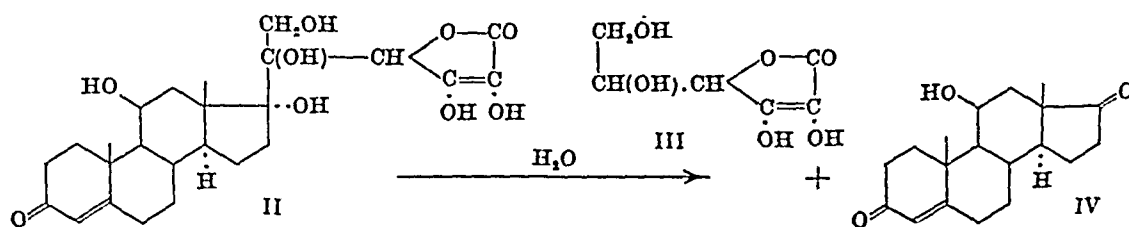
Compound	Group	Solubility in water	Survival test in rats	Survival test in adrenal- ectomized dog	$\text{Na}^+$ and $\text{Cl}^-$ retention in normal dogs
Deoxycorticosterone	$\text{C}_{21}\text{O}_3$	+	++++	+++	++++
Substance S	$\text{C}_{21}\text{O}_4$	++	++	?	?
Corticosterone		++	++	++	++
11-Dehydrocorticosterone		++	++	++	?
Substance M	$\text{C}_{21}\text{O}_5$	+++	+	+	-----*
Substance F		+++	+	+	-----*
Amorphous fraction	$\text{C}_{21}\text{O}_5(?)$	+++	+++++ (Kuizenga)	+++++ (Kendall)	+ (Kendall)
Compound	Group	Everse-de Freemery test	Ingles test	Diabeto- genic action	Anti- insulin test
Deoxycorticosterone	$\text{C}_{21}\text{O}_3$	++++	$\pm$	0	0
Substance S	$\text{C}_{21}\text{O}_4$	++	$\pm$	?	0
Corticosterone		++	+++	++	++
11-Dehydrocorticosterone		?	+++	++	?
Substance M	$\text{C}_{21}\text{O}_5$	+	++++	?	+++
Substance F		+	++++	++	+++
Amorphous fraction	$\text{C}_{21}\text{O}_5(?)$	+++ (Reichstein)	+ (Kendall)	$\pm$ (Kendall)	?

\* Negative retention = increased excretion.

fractions behave in such a way as to break the vertical sequences given by the six pure crystalline hormones. It appears likely, therefore, that suitably prepared concentrates may contain one or more unknown, possibly labile compounds, probably

belonging to the  $C_{21}O_5$  group, which are more active than any of the hormones yet isolated in a state of purity. Numerous, chemically labile, compounds involving variation of the side-chain have been synthesized, but in every case exhibited lesser activity than deoxycorticosterone in survival tests. There is evidence of synergism, e.g. between deoxycorticosterone and substance F, but only to a small degree.

Finally, reference must be made to the preliminary report [Lowenstein & Zwemer, 1946] which claims the isolation, from a concentrate, of 11-dehydrocorticosterone and two incompletely characterized substances, the combined activity of which equalled 80 % of the activity of the original material. One of the new compounds is possibly (II) and gives by mild anaerobic hydrolysis ascorbic acid (III). The other fragment (IV) has not been isolated but should be readily obtainable from substance U, recently synthesized by Sarett [1946] by oxidation with periodic acid.



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Prof. ZUCKERMAN, referring to the fact that of twenty-eight steroid compounds isolated from the cortex, only six have biological activity, asked whether Dr Shoppee believed that the transformations which required great chemical skill for their demonstration in the laboratory actually occurred in the adrenal cortex in response to the particular requirements of the body at any given time.

In replying, Dr Shoppee pointed out that there was no doubt that chemical transformations occur in nature much more easily than is possible in the laboratory, and that it is not known whether the twenty-eight substances isolated from the adrenal cortex are natural entities or artefacts, since it is possible that they represent degradations of true active principles.

Prof. YOUNG questioned whether two compounds present only in very small quantities in cortical extracts, and both with no activity in electrolyte metabolism, but potent in their effects on carbohydrate metabolism, could in fact be artefacts.

Prof. FRAZER, in support of this point of view, suggested that the natural secretions of a gland may well be those substances which are extracted in smallest amounts.

Methods of chemical preparation probably isolated the more stable compounds, but substances which were unstable, and whose action in the body would be limited for that reason, might have the more important biological function.

In reply, Dr Shoppee pointed out that the output of cortical substances may be considerable, and that the gland functions as a factory and not as a depository. The amount of material which can be extracted from it at any given moment bears no necessary relation to its production potential.

**The pituitary-adrenocortical relationship.** By M. REISS. *Biochemical and Endocrinological Department, University of Bristol*

The isolation and investigation of the properties of corticotrophic hormone fractions are very lengthy procedures. Since the size and weight of the adrenals may be increased not only by the active corticotrophic principle but also by toxic and so-called 'biologically inactive' impurities, which themselves rapidly mobilize corticotrophin, it is necessary to use hypophysectomized animals for biological tests of corticotrophin fractions. The change in weight of the adrenals of the hypophysectomized animals is, however, only rarely correlated directly with dose, since the adrenal is weighed during a period of progressive atrophy which starts on the first day after hypophysectomy and ends after 4 weeks.

Changes in the histological structure of the cortex provide a more definite index of corticotrophic activity [Reiss, Balint, Oestreicher & Aronson, 1936; Simpson, Evans & Li, 1943]. Disappearance of the sudanophilic substances after hypophysectomy can be restored by corticotrophic extracts. Dose-response curves may be obtained and compared with similar curves using a laboratory standard preparation [Carpenter, Macleod & Reiss, 1946]. No international standard of corticotrophin is actually available, but since the international standard preparation of lactogenic hormone contains a considerable amount of corticotrophin, it can be used as a laboratory standard.

This histological method is by no means ideal. The sensitivity of hypophysectomized animals changes from week to week, and about six times the amount of hormone used in the first week after removal of the pituitary is necessary 4 weeks later. It would be preferable to analyse the chemical changes induced in the adrenals by corticotrophin, since such changes, if constant in character, might serve as a basis for a more satisfactory biological assay method.

Sayers, Sayers, Tsan-Ying & Long [1945] believe that the ascorbic-acid content of the adrenal is considerably decreased 1 hr. after the injection of corticotrophic extracts, and claim that the decrease is proportional to the dose of hormone injected. Using hypophysectomized rats weighing 120-150 g., or infantile rats weighing 30-40 g., we have found this method very promising. It is very sensitive ( $\frac{1}{10}$  sudanophobic unit produces an ascorbic acid fall of 60 mg. %), and may prove suitable for the analysis of corticotrophic hormone in the body fluids. It should be pointed out, however, that corticotrophic hormone is apparently mobilized extremely quickly by different stimuli. For example, in normal non-hypophysectomized rats either intraperitoneal injection of a hypertonic solution, or ether narcosis, or 20 min. in

a cold room, decreases the ascorbic acid content of the adrenals by 30 %. This does not happen when hypophysectomized rats are used. Fewer animals and controls will, therefore, be needed if hypophysectomized animals are used for the test, and if a comparison is made between the two adrenals, one being investigated before injection of the corticotrophin, the other 1 hr. after.

Definite proof of corticotrophic action, that is, the mobilization of cortin, has been provided by investigation of the cortin content of adrenals, blood and urine after treatment with corticotrophic hormone. Use was made of the reducing properties of the side-chains in order to determine the nature of the cortical steroid compounds [Hemphill & Reiss, 1947].

Considering the many compounds produced by the adrenal cortex, it is quite possible that several corticotrophic fractions exist. Certain extracts derived from the serum of pregnant mares are said to increase the weight of the adrenals of hypophysectomized animals without influencing the distribution of lipoids [Golla & Reiss, 1942]. Further, it is well known that the potency of corticotrophic extracts in increasing adrenal weight may decrease considerably with progressive purification in parallel with an increasing capacity to restore the distribution of sudanophilic lipid in the adrenals. Size, lipid distribution and hormone production might well be three entities dependent upon different corticotrophic fractions of the pituitary.

Various methods are available both for the preparation of crude corticotrophic extracts and for their purification [Lyons, 1937; Reiss & Golla, 1945]. Their separation from gonadotrophic, thyrotrophic and growth hormone extracts is made possible by their solubility in higher concentrations of alcohol and acetone, and by their greater resistance to heat and acid. According to several authors, corticotrophic extracts may also be prepared by ultrafiltration or dialysis. In our experience, however, the total corticotrophic activity of crude extract has never been transmitted to the ultrafiltrate or dialysate, even after most exhaustive electrodialysis. Over 50 % of the active substance has always remained in the residual liquor or on the filter. It is not impossible that different corticotrophic hormone fractions (one with a molecular weight of 3000-6000 and another of 20,000-40,000) are separated in this manner.

Li, Simpson & Evans [1940], and Sayers, White & Long [1943] both claim to have isolated a pure adrenocorticotrophic hormone. They have applied different fractionation procedures to pituitaries of different species, and have obtained nearly identical end-products. The pure hormone, prepared essentially by isoelectric precipitation and salt fractionation, is claimed to be a substance with a molecular weight of 20,000. It is heat stable at 100° C. at pH 7.5 or in N/10-HCl, but activity is lost by heating in N/10-NaOH solution. It appears to be a polypeptide which is destroyed by trypsin but not by pepsin. Unlike the gonadotrophins, it contains no carbohydrate group. It contains 2.33 % sulphur, which may be accounted for by the presence of 2.3 % cystine and 7.19 % methionine. Inactivation experiments suggest that, as in the case of other anterior lobe hormones, certain amino groups are essential for its biological activity, while acetylation experiments suggest that phenolic hydroxyl groups are also concerned [Li *et al.* 1946].

As is well known, the anterior lobe of the pituitary is not in complete control of the activity of the subordinate glands, but only regulates their function in accordance

with the needs of the body. Adrenal function is considerably diminished after hypophysectomy, but animals survive the operation for a year or more, whereas they would die within a few days if all cortical tissue were removed.

Corticotrophic extracts apparently mobilize all the compounds produced in the adrenal cortex. Even the excretion of 17-ketosteroids is increased after injection of corticotrophic extracts [Hemphill, Macleod & Reiss, 1942]. Since, however, it is not yet known whether all cortical compounds have been chemically isolated, nor whether all corticosteroids isolated or synthesized are really produced by the adrenal cortex, it is possible for certain effects of corticotrophins to differ from the action of injected adrenal corticosteroids. The therapeutic results achieved with corticotrophic extract may indeed surpass those of cortin extracts or of chemically isolated corticosteroids.

In the rat carbohydrate metabolism is considerably disturbed after hypophysectomy. The animal loses much of its ability to retain sugar, which it wastes by increased combustion. Thus, only 29–36 % of glucose given orally is burned by a normal rat, whereas eight days after hypophysectomy the same rat will burn 52–62 %. After a few days' treatment with corticotrophic hormone the animal again burns only 25–47 % [Reiss, Kusakabe & Budlowsky, 1938]. This action might be due to the mobilization of 17-hydroxy-11-dehydroxycorticosterone, which causes appreciable increases in muscle glycogen. Russell [1943] has also found that cortical extracts inhibit glucose utilization in hypophysectomized and adrenalectomized rats.

Ingle, Winter, Li & Evans [1945] force-fed rats with carbohydrate, and claim to have produced glycosuria after 2 days' treatment with purified corticotrophic extract. This effect might be due to the mobilization of corticosterone or 17-hydroxycorticosterone [Ingle, Sheppard, Oberle & Kuizenga, 1946].

Corticotrophic hormone not only increases the glycogen content of the carbohydrate stores but also the total fat content of the body [Reiss, Epstein & Gothe, 1937]. In some incompletely hypophysectomized animals, the whole sella is very soon filled with compensatory hypertrophied pituitary tissue. Such animals usually look particularly well, and their total fat content may be as much as 80 % more than normal. The size of their adrenals is also considerably increased. It is noteworthy that this state may develop within a fortnight of the partial hypophysectomy, and it seems possible that it is due to the mobilization of a cortical compound responsible for fat deposition. Certain forms of adiposity appear to be elucidated by this observation. For example, we have repeatedly found increased corticotrophic hormone excretion, while Anderson & Haymaker [1944], and recently Heard, Sobel & Venning [1946], have found increased excretion of corticosteroid in Cushing's syndrome. Increased 17-ketosteroid excretion in the syndrome is already a well-established fact.

The protein loss seen in some sufferers from the disease may be an indirect effect of the activity of corticotrophic hormone. An increase in urinary nitrogen excretion, which is manifest on the second day of treatment with a purified corticotrophic hormone fraction, has been claimed by Evans's laboratory, and this fraction is supposed also to counteract the action of pituitary growth hormone [Marx, Simpson, Li & Evans, 1945].

Data about corticotrophic activity in mobilizing desoxycorticosterone or corticosteroids active in water and salt metabolism are still scarce. A renotropic action has been claimed [Reiss, 1944], but Simpson, Li & Evans [1946], also using high doses of



a purified corticotrophic hormone, failed to find any such action. Using unpurified anterior pituitary extracts, Hall & Selye [1945] produced nephrosclerotic changes similar to those found after treatment with desoxycorticosterone. The changes could be prevented by high carbohydrate diets or by ammonium chloride or calcium chloride.

Crude corticotrophic hormone extracts decreased the thyroid activity and the blood iodine content of dogs [Reiss & Peter, 1938]. This observation reveals the antagonistic relation between thyroid and adrenals.

The different metabolic functions of the adrenocorticotrophic hormone provide an obvious basis for the important role this hormone plays in maintaining and, in emergencies, increasing the resistance of the body against a variety of disturbances.

It has been shown that the capacity of the reticulo-endothelium to store lithiocarmine is increased 1 hr. after injection of a corticotrophic hormone fraction [Reiss & Gothe, 1937]. White & Dougherty [1945] have recently found that injection of corticotrophic extract is also followed by a rise of the serum proteins, largely due to a rise in the gamma globulin fraction containing the antibodies, and that the rate of antibody release is under the control of corticotrophic hormone. A lymphopenia occurs after treatment with corticotrophic hormone and corticosteroids. Changes in weight and size of thymus and lymph nodes, and a decrease in the lymphocyte content of the thoracic duct lymph, are also well-established observations [Yoffey, Reiss & Baxter, 1946].

The output of corticotrophic hormone increases rapidly as soon as one (or both) adrenal is removed. The compensatory hypertrophy of the remaining adrenal after unilateral adrenalectomy is clearly due to increased production of corticotrophic hormone, since it does not take place after hypophysectomy.

Hypertrophy of the adrenal occurs very often, and under very different conditions, and it may safely be assumed that it is primarily due to an increased demand for one of the adrenal cortex hormones. If animals are kept in the cold, for example, the weight and cortin content of the adrenals rapidly increases. The corticosteroid content of the blood also rises by several hundreds per cent. Tepperman, Engel & Long [1943] have comprehensively reviewed the conditions for adrenal cortical hypertrophy, and it seems well established that most of the forms of hypertrophy are secondary to a primary stimulation of the pituitary anterior lobe.

The concentration of corticotrophic hormone in the urine has also been studied. It increases, together with that of the corticosteroids, during pregnancy [Reiss, Peglar & Golla, 1946], and in Cushing's syndrome, as well as in diabetes and after insulin coma [Bartelheimer & Cabeza, 1942].

Very little is known about the nervous mechanisms that stimulate or inhibit the production of corticotrophic hormone by the pituitary. Excretion is sometimes increased after prefrontal leucotomy and so is the excretion of 17-ketosteroids [Hemphill *et al.* 1942]. It is probable that the production of the corticotrophic hormone is influenced by emotional states. In the syndrome of anorexia nervosa, for example, cortin excretion is very much decreased (0.1–0.2 mg. in 24 hr.) and so is the excretion of 17-ketosteroids (0.5–4 mg. in 24 hr.) [Reiss, 1943]. When such cases recover, corticotrophic hormone and 17-ketosteroid excretion increase to the normal level. Sometimes, however, such patients swing to the opposite extreme, and produce

considerably increased amounts of corticotrophic hormone. Ketosteroid excretion also increases, while the patient becomes abnormally fat [Reiss, 1943].

One begins to realize, when surveying the whole field of the pituitary-adrenal relationship, how important its final clarification is for the analysis of a multitude of body regulations. The main function of corticotrophin is the conservation of energy, and the support of the organism in complying with increased demands. It is thus a vital factor in the maintenance of the body.

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Mr CHANCE inquired whether anything is known about the interrelation of the steroids and ascorbic acid metabolism, and whether it is likely that the ascorbic acid used up by these hormones is a significant part of the body's ascorbic acid content. He also asked what effect corticotrophin had on the adrenals of scorbutic animals.

Dr Reiss informed the meeting that experiments on the effect of corticotrophin on the adrenals of scorbutic animals were in progress.

#### The influence of the adrenal cortex on metabolism.

By F. G. YOUNG. *University College, London, W.C. 1*

The pioneer investigations carried out by Addison and others showed that the adrenal glands are essential to life, but the lack of any means of extending the life of adrenalectomized animals at first prevented a clear determination of the nature of the functional upset following adrenalectomy. In consequence, until the brilliant work of

Swingle and Pfiffner provided potent adrenal extracts, only a vague 'detoxification' action was ascribed to the adrenal glands. The view then developed that the function of the adrenal cortex was to control electrolyte balance, particularly with respect to sodium and potassium. This suggestion was supported by the demonstration that adrenalectomized animals could be kept alive almost indefinitely by suitable adjustment of the sodium and potassium content of the food. Later, the acceptance of Britton and Silvette's belief that the adrenal cortex is of particular importance in carbohydrate metabolism began a phase in the history of cortical physiology in which the elucidation of further facts has continued to render untenable each successive theory that has so far attempted to correlate the various aspects of adrenal-cortical function.

Before surveying some of the vast amount of evidence now available, an important limitation of the prevailing experimental approach should be considered. In experiments designed to elucidate the action of hormones one is concerned not only with the effect of the hormone on the animal, but also with the interaction of the animal and the hormone. Adrenalectomy, for example, induces varying degrees of anorexia, which itself may result in widespread metabolic changes not directly related to adrenal-cortical function. Paired-feeding control experiments are therefore of the greatest importance, even though they have been somewhat neglected in the past. Again, changes in metabolic pattern may develop, sometimes slowly, in an animal treated with adrenal steroids, so that the results may be quite different according to the duration of the experiment. It is always possible that metabolic transformations may partially convert one administered adrenal steroid into another.

### *The influence of adrenalectomy on electrolyte and water balance*

It is probable that one of the primary actions of adrenal cortical steroids is to influence the renal excretion of  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  ions, probably by affecting tubular reabsorption. In untreated adrenalectomized animals there is usually an excessive urinary excretion of  $\text{Na}^+$  and  $\text{Cl}^-$ , with diminished excretion of  $\text{K}^+$ . The loss of  $\text{Na}^+$  and  $\text{Cl}^-$  from the kidney results in a diminished concentration of these ions in the blood plasma, and is associated with an increased loss of water from the blood into the urine. There is also a loss of  $\text{Na}^+$  from the muscle cells, and a rise in the  $\text{K}^+$  content of the cells of the tissues and of the blood plasma. The early changes in electrolyte balance found in rats 3-6 days after adrenalectomy are well illustrated by the recent comprehensive results of Conway & Hingerty [1946].

Calculation of the concentration of  $\text{Na}^+$  and  $\text{K}^+$  actually within the muscle fibres [Conway & Hingerty, 1946] showed that there is no correspondence between the  $\text{K}^+$  gain and the  $\text{Na}^+$  loss. Calculation also showed that there is a fall in the total number of such characteristic constituents as  $\text{Na}^+$  and hexose phosphates, which the cell retains and which do not diffuse through its membrane, while simultaneously the sum of their negative charges rises. It also appears that there are other unascertained changes in basic muscle constituents.

Conway & Hingerty's observations show a striking increase in  $\text{Mg}^{++}$  content of both plasma and muscle. The rise in muscle  $\text{Mg}^{++}$  may well contribute to the asthenia which follows adrenalectomy, especially since  $\text{Mg}^{++}$  is known to inhibit adenosine-triphosphatase.

Although the accumulation of potassium in the body can lead to death, the rise in blood  $K^+$  which follows adrenalectomy cannot be regarded as the sole cause of death, since a rise of blood  $K^+$  equal to that found in adrenalectomized animals can be induced by other means without fatal results. Likewise the depletion of  $Na^+$  cannot be regarded as the sole cause of death from adrenal failure. It seems that no single cause can account for the changes in electrolyte balance which follow adrenalectomy. A failure of the kidney tubules to reabsorb  $Na^+$  may be a factor, while it is possible that the glomeruli also secrete  $K^+$  and  $Mg^{++}$  less effectively. Although the idea has the merit of simplicity, it would be dangerous, however, to assume that the changes in the electrolyte constitution of the muscles result directly from those of the blood plasma and tissue fluids. It is certain that the rise in extracellular  $K^+$  is not the result of leakage of intracellular  $K^+$ , as was at one time considered possible, since the  $K^+$  content of the interior of cells is now known to rise rather than to fall.

Initially the abnormalities in electrolyte distribution are associated with diuresis, but later the ability of the animal to excrete administered water may be greatly diminished, and there is usually an exaggerated susceptibility to water intoxication. Oliguria resulting from a diminished water intake, associated with a general failure of kidney function, may be a terminal symptom.

A fact which cannot be overlooked in any general theory of the influence of the adrenal cortex on electrolyte metabolism is that adrenalectomized animals remain in good health and even grow at a normal rate provided they are given sufficient extra-dietary  $NaCl$ . This simple fact provides a serious check to many otherwise plausible theories.

#### *Influence of adrenalectomy on carbohydrate metabolism*

The glycogen stores of a well-fed adrenalectomized animal may be normal, but they rapidly diminish as a result of starvation, and may be very much below normal because anorexia is common in advanced adrenal insufficiency. The abnormal depletion of glycogen has been ascribed both to an excessively high rate of carbohydrate oxidation during starvation, and also to diminished glyconeogenesis from protein in the liver. Animals exhibiting adrenal insufficiency are unable to store administered carbohydrate (in the form of glycogen) as rapidly as normal animals, particularly in the liver. An excessively high R.Q. during the feeding of glucose shows that an undue proportion of the available carbohydrate is undergoing oxidation. It is a remarkable fact that these profound changes in the storage and oxidation of carbohydrate in the adrenalectomized animal can, in the rat at least, be almost entirely alleviated by giving extra  $NaCl$  [Anderson, 1943], a substance for which the adrenalectomized rat has an abnormally large appetite.

Although a striking alleviation of experimental diabetes often follows adrenalectomy, recent careful experiments with the alloxan-diabetic rat [Janes, Dawson & Myers, 1946], in which the paired-feeding technique was employed, have shown that the diminution in urinary and blood-sugar level is due to the lowered food intake rather than to adrenalectomy *per se*. Nevertheless, after starvation for 24 hr., the carbohydrate stores of the adrenalectomized rats were less than one-sixth those of the control diabetic animals.

Since the anti-insulin activity of anterior pituitary extracts can be demonstrated in adrenalectomized rabbits maintained on  $NaCl$  [Himsworth & Scott, 1938], and since

a diabetes-inducing action of anterior pituitary extract has been demonstrated in the partially depancreatized-adrenalectomized dog maintained on NaCl [Houssay, 1942; Houssay, Foglia & de Pasqualini, 1946], it is certain that the influence of the anterior pituitary gland on carbohydrate metabolism is not exerted solely by way of the secretions of the adrenal cortex. Nevertheless, under some conditions adrenocorticotrophin can exert a diabetogenic action in the rat [Ingle, Li & Evans, 1946].

The hypoglycaemic action of a small dose of insulin is greatly exaggerated in adrenalectomized animals, and a profound degree of hypoglycaemia may develop in those which are anorexic. There is no certain evidence, however, that the insulin content of the pancreas of adrenalectomized animals is abnormal [Haist, 1944], and it seems probable that the abnormal sensitivity of the adrenalectomized animal to the hypoglycaemic action of insulin is not cured by salt therapy. Hence the unstable blood-sugar level of some human patients with Addison's disease. There is good evidence that the absence of the adrenal medulla is not the sole cause of the insulin hypersensitivity.

Despite the serious abnormalities in carbohydrate metabolism which follow adrenalectomy, it is not possible to ascribe the death of the adrenal-insufficient animal solely to these disturbances.

#### *Influence of adrenalectomy on protein metabolism*

When an untreated adrenalectomized rat is starved, the urinary nitrogen excretion is about 25 % below that of control animals. Recently, however, Ingle & Oberle [1946] have demonstrated that during prolonged experiments the starving adrenalectomized rat given 1 % NaCl to drink excretes as much non-protein nitrogen in the urine as do control animals. In other experiments, involving the forced feeding of adrenalectomized rats receiving NaCl, little or no abnormality in protein catabolism could be detected. During the development of the symptoms of adrenal insufficiency the blood-urea content tends to rise, due, probably, to general depression of kidney function rather than to any specific effect on protein metabolism.

In adrenal insufficiency the plasma-protein concentration may rise, in association with the diminution in blood volume, but the albumin fraction of the plasma-albumin content is not completely restored by the administration of NaCl. In the experiments of Leatham [1945], however, pair-fed control rats exhibited a similar diminution in plasma protein, so the effect in the salt-treated adrenalectomized animal could be ascribed to the slight diminution in food intake.

It is well known that chronic adrenal deficiency, particularly in human beings, may be associated with persistence of the thymus gland, and that the thymic involution which normally follows Selye's adaptation reaction is not observed in adrenalectomized animals. Antibody formation is depressed and the blood contains fewer polymorphonuclear leucocytes [Dougherty & White, 1944]. There is also an increase in the combined leucocyte and lymphocyte count, which is greater than could be accounted for by the observed increase in the concentration of the blood [Dougherty & White, 1944]. It thus appears that the adrenal cortex exerts an important influence over lymphoid tissue in general, and in particular over the release of antibody from lymphocytes [Dougherty, Chase & White, 1945].

*Influence of adrenalectomy on fat metabolism*

Here the results are much less clear-cut. Less fat is stored in the livers of adrenalectomized animals, and in general the development of fatty liver does not occur so readily. It is possible that the rate of oxidation of fat is depressed, but it is difficult to obtain unequivocal evidence. Experimental ketonuria can often be diminished in intensity by adrenalectomy, but this effect may be due, in part at least, to a rise in the kidney threshold for ketone bodies.

*Influence of adrenalectomy on general metabolism and growth*

After adrenalectomy the B.M.R. may at first be unchanged, but later it may fall. Chronic adrenal insufficiency depresses growth in the young rat, and adrenalectomized rats given 1 % NaCl to drink increase in weight at a normal rate. In paired-feeding control experiments Roy [1947] has shown that the bodies of adrenalectomized rats receiving NaCl contain, after 3 weeks, 15 % more fat and 5 % more protein, but about 60 % less carbohydrate than those of control animals, which suggested that the metabolic rate had been significantly subnormal in these animals.

Lactation is inhibited in adrenalectomized animals, but it is unlikely that the adrenal cortex secretes a specific lactogenic substance.

*General conclusions regarding the effects of adrenalectomy on metabolism*

The metabolism of the salt-treated adrenalectomized rat is remarkably normal provided that the dietary and environmental conditions are stable. Species other than the rat have not been so well investigated, but experiments on particular aspects of the metabolism of the salt-treated adrenalectomized rabbit or dog have given comparable results. It must be pointed out, however, that the rat frequently has accessory cortical tissue which is not necessarily removed at operation, and it is possible that survival and activity after adrenalectomy are thereby influenced. Nevertheless, it is clear that the pharmacologically powerful adrenal steroids are not so important for the maintenance of a 'quiet life', under some conditions at least, as might otherwise be expected.

There seems to be no simple general theory which satisfactorily co-ordinates all the various aspects of the effects of adrenalectomy, and at present judgement must be suspended as to the nature of any general pattern underlying the diverse data that are available.

*Assay of adrenal-cortical extracts*

The foregoing discussion clearly emphasizes the importance of controlling the dietary and environmental conditions in experiments concerning the function of the adrenal cortex. In assays of physiologically active adrenal-cortical substances the conditions must be equally rigidly controlled.

The maintenance of the adrenalectomized dog or rat in good health has been employed as the primary criterion of activity of adrenal extracts, while the retention of  $\text{Na}^+$  and  $\text{Cl}^-$  in a normal or in an adrenalectomized dog has been also utilized. Two methods of assay which have been extensively used are the 'Everse-de Fremery' work test, based on the height of the contractive response in the stimulated muscles of the extract-treated adrenalectomized rat, and the 'Ingle' work test, which utilizes the

total amount of work the muscles of the treated adrenalectomized rat are capable of performing when stimulated to exhaustion. Another widely used method is based on the ability of adrenal preparations to protect the adrenalectomized rat against the otherwise lethal effects of a low environmental temperature, while another utilizes one of the numerous effects of adrenal extracts on carbohydrate metabolism.

Table 1. *Comparative physiological activities of adrenal preparations*

(The figures indicate the order which the seven preparations occupy with respect to activity in the various tests; the most active substance is designated 1, and the least active 7.)

Substance	Life maintenance test	'Everse-de Fremery' work test	'Ingle' work test	Influence on carbohydrate metabolism	Na <sup>+</sup> and Cl <sup>-</sup> retention in normal dog
Corticosterone	3	3	3	3	3
17-Hydroxycorticosterone	4	5	1	1	?
11-Dehydrocorticosterone	4	?	4	4	3
17-Hydroxy-11-dehydrocorticosterone	4	5	1	1	?
11-Deoxycorticosterone	2	1	6	5	1
17-Hydroxy-11-deoxycorticosterone	7	3	6	6	?
Amorphous fraction	1	2	5	?	2

Table 1 summarizes the available information regarding the relative activities of the six active adrenal steroids and of the amorphous fraction. It suggests the following conclusions: (1) activity in the 'Everse-de Fremery' test approximately parallels that in the 'life maintenance' assay; (2) results with the 'Ingle' work test parallel those that concern carbohydrate metabolism; (3) the presence of an oxygen atom (hydroxyl group or ketonic oxygen) at C<sub>11</sub> of the steroid nucleus increases activity with respect both to the 'Ingle' work test and to carbohydrate metabolism; (4) the presence of a tertiary hydroxyl group at C<sub>17</sub> diminishes activity in the 'life maintenance' test and in the Na<sup>+</sup> and Cl<sup>-</sup> retaining assays. Indeed, 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone exhibit a tendency to facilitate the excretion of Na<sup>+</sup> and Cl<sup>-</sup> in a short-term test, although in longer term tests activity in retaining these ions may occur [Ingle, 1944].

#### *Influence of adrenal steroids on oxidative metabolism*

When adrenal steroids induce a rise in the glycogen stores, or induce or exacerbate a diabetic condition, the nitrogen excretion of the treated animal rises initially, though the negative N balance may not be maintained in a long-term experiment. It is assumed that glyconeogenesis from protein is a contributory factor in the accumulation of the extra glycogen or glucose. Nevertheless, if the classical data relating to the conversion of protein to carbohydrate (100 g. of protein yields 58 g. of carbohydrate) be adopted, it is not possible to account for all of the extra carbohydrate which appears as a result of adrenal steroid treatment. It seems that the adrenal steroids diminish the rate of utilization of carbohydrate by the peripheral tissues, and that this is an important factor in the antagonism of many adrenal steroids to the hypoglycaemic action of insulin. Ingle has recently found that when diabetes is induced in normal rats by adrenal steroids, large doses of insulin, sufficient to bring the hyperglycaemia and glycosuria under control, do not reduce urinary nitrogen excretion to the normal level. It therefore seems unlikely that the enhanced rate of formation of carbohydrate from protein, induced by adrenal substances, can be secondary to inhibition of carbo-

hydrate oxidation brought about by such treatment. Since the respiratory quotient is depressed as a result of the administration of adrenal steroids, it appears possible that the depression of carbohydrate oxidation may be associated with an increased combustion of fat.

If the adrenal cortical secretions directly stimulate the breakdown of intracellular protein, it seems paradoxical that the presence of the adrenal cortex or of its secretions is necessary for the manifestations of the full growth-promoting activity of anterior pituitary extract, particularly in view of the fact that the administration of excess adrenal steroids inhibits the growth of young rats. It may be pointed out, however, that the incorporation of exogenous amino-acids into the tissues may be assisted by a limited stimulation of the catabolism of tissue protein, although undue enhancement of protein catabolism would obviously prevent or depress growth.

The administration of adrenal steroids with an oxygen atom at  $C_{11}$  causes rapid regression of the thymus in rats, together with an absolute lymphopenia. Since an increased production of antibodies has been demonstrated in animals treated with adrenal extract but not with deoxycorticosterone acetate, it seems probable that certain of the corticosterone series control the release of antibodies from the lymphocytes.

#### *The influence of the adrenal cortex on enzyme systems*

**Phosphorylation.** The importance of phosphorylation mechanisms with regard to hormone action has been recently emphasized by the experiments of Price, Cori & Colowick [1945] on the enzyme hexokinase. This enzyme catalyzes the transfer of a phosphoric-acid group from adenosine-triphosphate to glucose, yielding adenosine-diphosphate and glucose-6-phosphate. Cori and his colleagues have demonstrated that the activity of this enzyme is inhibited *in vitro* by anterior pituitary extract, and that this inhibition is annulled *in vitro* by insulin. The inhibitory action of the anterior pituitary extract is enhanced by crude adrenal-cortical extract *in vitro* [Price, Slein, Colowick & Cori, 1946], though the latter appears to have no inhibitory action in the absence of pituitary extracts. Surprisingly enough, the action of the crude adrenal extract was traced not to any of the crystalline steroids, but to the amorphous fraction. This is particularly unexpected in view of the fact that the amorphous fraction has no obvious action on carbohydrate metabolism *in vivo*.

The data of Conway & Hingerty [1946] regarding the influence of adrenalectomy on the hexose-phosphate content of skeletal muscle are probably of importance here, though once again a failure to provide any simple generalization must be recorded. It is reasonable, however, that in the presence of unleashed hexokinase activity in the adrenalectomized rat, glucose-1-phosphate should accumulate, though this presumably would be secondary to an initial enhanced rate of formation of glucose-6-phosphate.

The activity of other enzymes, arginase [Fraenkel-Conrat, Simpson & Evans, 1943; Folley & Greenbaum, 1946], phosphatase, and various oxidases is usually altered by adrenalectomy, but the significance of the findings is still obscure.

#### *General conclusions*

It seems possible that the adrenal hormones, like enzymes, are not essential for the processes of the body, but that they catalyze mechanisms, which would proceed more slowly in the absence of the hormones. It seems that two important processes in-



fluenced by adrenal hormones are the mobilization of the body's protein stores, and the inhibition of the action of hexokinase in facilitating the utilization of glucose, as opposed to that of glycogen. But at present no simple theory can reconcile all of the large mass of apparently incompatible data.

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Dr FOLLEY agreed that a criticism of work on the responses of the body to different cortical steroids is that the electrolyte content of the diet has rarely been taken into due consideration. The possibility that alterations in the electrolyte intake of experimental animals may influence the relevant responses to different cortical steroids, as suggested by Kendall, affected work now being pursued in his laboratory on the effects of the adrenal cortex on lactation. It is known that lactation is practically suppressed after adrenalectomy, and Kendall and his associates claim that it can be restored only by the 11-oxygenated corticosteroids. On the other hand, Dr Folley found that this compound is relatively inactive, while DOCA gave the best results. It seems quite possible that the electrolyte content of the respective diets might be responsible for the discrepancy between the two experiments.

Prof. FRAZER emphasized the different metabolic paths taken by short- and long-chain fats, and agreed with Prof. Young in stressing the importance of strict control of the constituents of the diet. He also wanted to know if a shortage of blood cholesterol had any effect on the production of adrenal hormones. Prof. Young replied that the whole question of the influence of the adrenal cortex on fat metabolism needs to be investigated anew.

Dr SHOPPEE referred to an analogous case in which Bloch fed a labelled deuterio-cholesterol to a woman in the eighth month of pregnancy, and was able to show that, on the basis of the isotopic content of the excreted pregnane-diol, something like two-thirds was derived from the marked cholesterol.

**The adrenal cortex in shock and stress.** By G. UNGAR.

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A mass of experimental evidence suggests that the adrenal cortex plays a prominent part in protecting the body against various harmful environmental stimuli. In interpreting the facts it is essential to avoid confusing the problem through teleological thinking.

*Experimental facts*

These can be classed in three groups: (1) lowered resistance of adrenalectomized animals to various stimuli, (2) morphological changes in the adrenals after exposure to such stimuli, and (3) effect of adrenal hormones on resistance.

(1) *Adrenalectomy.* It has been known since 1897 [Boinet] that adrenalectomy increases the sensitivity of animals to a number of toxic substances. The first well-controlled and correctly interpreted observations are those of Dale [1920] who showed that adrenalectomized cats are more than sixty times more resistant to histamine than normal animals. Dale's work was later confirmed in various other species by a number of investigators.

It was subsequently shown that adrenalectomized animals (in most cases rats) become more susceptible to the toxic action of a bewildering variety of drugs; e.g. morphine, papaverine, codeine, veratrine, atropine, adrenaline, digitoxin, acetonitrile, cyanides, lead, arsenic, curare, certain snake venoms and bacterial toxins.

A number of other conditions have been shown to be associated with a higher mortality in adrenalectomized animals; e.g. trauma [Freed, 1933], anaphylactic shock [Kepinow, 1922], intestinal obstruction [Cutting, 1929], exposure to extreme temperatures, acute starvation, and anoxia by lowered atmospheric pressure [Evans, 1934]. Two points need to be stressed about this class of observations:

(a) In most cases they were made on adrenalectomized rats maintained with NaCl.

(b) The protection afforded by the adrenal cortex is quantitative. Hechter, Krohn & Harris [1942] found that with a standardized muscle trauma the mortality rate is:

Bilateral adrenalectomy	100 %
Unilateral adrenalectomy	75 %
Normal animal	0

This 'quantitative' effect was confirmed in anaphylactic shock [Kepinow, 1922], and many other cases.

(2) *Morphological changes.* Brown Séquard [1852] was the first to observe that the adrenals react by hyperaemia and haemorrhage to trauma inflicted on a distant part of the body.

Haemorrhage in the adrenals has been observed in various conditions; e.g. injection of diphtheria toxin [Roux & Yersin, 1889; Behring, 1890].

These and other similar findings led to the theory, now abandoned, that a variety of shock conditions were the expression of an adrenal deficiency.

More important are the opposite changes observed in the adrenals after administration of noxious stimuli, and which are characterized by hypertrophy of the gland and proliferation of the cortical secretory elements. The question has been reviewed by Tepperman, Engel & Long [1943], who point out that hypertrophy is associated with

most of the conditions to which adrenalectomized animals show an increased sensitivity. Among the stimuli which have not yet been investigated in adrenalectomized animals, but which may throw light on the mechanism of adrenal hypertrophy, are conditions which (a) raise the metabolic rate (experimental hyperthyroidism), (b) increase carbohydrate utilization (insulin, muscular exercise), or (c) increase protein catabolism (phlorizin diabetes, high protein diet).

All stimuli act through the pituitary, at least in mammals, and adrenal hypertrophy does not occur after hypophysectomy.

There is no certain evidence that hypertrophy means increased secretory activity of the gland, but there is an increased urinary elimination of cortin-like substances in cases where hypertrophy is known to occur.

(3) *Action of cortical hormones.* The data can be classed into two groups:

(a) Restoration of normal resistance in adrenalectomized animals. The bulk of the data show that resistance to various stimuli, impaired by adrenalectomy, can be restored by whole cortical extracts, cortin or the so-called sugar-active cortical steroids. It is improbable that desoxycorticosterone has any effect except in the case of resistance to cold.

(b) There is sharp disagreement about the possibility of raising resistance above the normal level by administration of cortical hormones. Divergent results are probably due to the nature of the product used (DOCA being generally without effect), and even more to differences in dosage. Moreover, considering the enormous output of cortical hormone that seems to occur naturally, the doses that have been used in most experiments and clinical trials were insignificant. The question will only be answered when large amounts of hormones other than DOCA are generally available.

### *Interpretation*

In the light of the facts just summarized, one may conclude that normal animals possess a mechanism, or rather a set of mechanisms, enabling them to resist certain 'aggressions'. These mechanisms depend very largely on the integrity of the adrenal cortex.

*Adaptation syndrome.* It has been known since 1911 that the administration of certain substances induces a temporary increase in resistance to themselves and to other non-related conditions. This fact, known under a variety of names, has been elaborated by Selye during the past ten years as a general synthesis under the name of alarm reaction or general adaptation syndrome.

Selye [1946] describes three main stages in the development of the adaptation syndrome: the alarm reaction proper, the adaptation phase and the exhaustion phase. The alarm reaction occurs independently of the adrenal cortex, but most of the symptoms of the adaptation phase cannot be produced in adrenalectomized animals.

In general, adaptation can be defined as a rise in the threshold of action of stimuli following the application of the same or another stimulus. The best known case is immunity which, however, is quite independent of the adrenal cortex. It is also known that adaptation can be produced in isolated organs and even isolated cells. It is therefore very important to distinguish between adaptation processes that are mediated through the adrenal cortex and those that are not. The adaptation syndrome, as originally described by Selye [1937], is probably one of the fundamental

modes of reaction of the organism to such stimuli as tend to change the constancy of its internal environment. To-day the study of the phenomenon needs to be extended more in depth than in the extent of its manifestations.

*Mode of action of the adrenal cortex.* It is unlikely that a single mechanism can explain all the facts that have been referred to. There is, for example, no evidence that any cortical hormone can neutralize any single toxic substance, while there is some evidence that several mechanisms are set into motion, regardless of the need for all of them.

Various components of the adaptation syndrome correspond to the functions of the adrenal cortex:

(a) The relative inefficiency of DOCA in alleviating conditions of stress suggests that changes in electrolyte metabolism probably have a limited role.

(b) Changes in carbohydrate and protein metabolism probably play a more important part. In anoxia, for example [Lewis, Thorn, Koepf & Dorrance, 1942], the carbohydrate stores of the body are depleted by increased sugar utilization. Cortical hormones correct this depletion by slowing down glucose utilization, and by replacing the lost carbohydrates through stimulation of gluconeogenesis from proteins.

(c) A very important component of the adaptation syndrome is the involution of the thymus and the lymphatic system. Adrenalectomy causes hyperplasia and noxious stimuli give rise to atrophy of the tissues (a response that is prevented by adrenalectomy). Recent work by White & Dougherty [1945] shows a correlation between involution of the lymphatic system, lymphopenia, increase in circulating globulins, and rise in antibody titre. This does not mean that the adrenal cortex determines the acquisition of immunity, but that it may control the output of already formed antibodies. As immunological processes do not seem to take part in the adaptation syndrome, the significance of lymphatic involution is, however, not yet clear.

(d) I have been able to show that the spleen also plays a part in the adaptation syndrome. Noxious stimuli shorten bleeding time, increase capillary resistance, and reduce capillary permeability. All these responses are prevented either by hypophysectomy, adrenalectomy, or splenectomy, and are restored by corticotrophic hormone, whole cortical extract, and suitable extracts of the spleen. Among the various cortical steroids tried, DOCA, corticosterone, Reichstein's F, J, K, M and U substances were inactive, A and S were slightly active, and the only substance which could quantitatively account for the action of whole cortical extract was the  $\Delta^4$ -pregnene-17-( $\beta$ ):20-( $\beta$ ):21-triol, 3-one, one of the more recently isolated cortical steroids.

The active splenic substance has been isolated in a very pure form, and its chemical analysis is being carried out by Prof. Hirst in Manchester. This substance, when injected into guinea-pigs that had been submitted to an 'alarming' stimulus, prevented the formation of the haemorrhagic lesions but had no effect on the other symptoms of the alarm reaction.

It is therefore reasonable to assume that the mechanism of adaptation is made up of a number of processes each correcting a particular type of pathological reaction. The best example of a particular process dealing with a particular stimulus is the mechanism by which the adrenal cortex controls the action of histamine. Thus adrenalectomy in rats is associated with a considerable increase in the histamine

content of the tissues. This can be reduced to the normal level by high doses of cortical extract and also by DOCA. These changes are due to variations in the activity of histaminase, which is apparently activated by some cortical product.

I have observed that there is a reduction in the amount of histamine that can be released from blood cells during the process of adaptation. This reduction can be inhibited by adrenalectomy, and is restored by adrenal extracts (but not DOCA). The phenomenon may be the same as that described by Rose & Browne [1941], and the apparent inhibition of histamine release may in fact be no more than the destruction of histamine by histaminase as soon as it is liberated. My own view is that it represents a different process. Histamine, we now know, is liberated through the action of a proteolytic enzyme normally held in check by an inhibitor. In certain pathological conditions the enzyme is set free, and is able to split off histamine from some protein substrate. It is thus possible that the adrenal cortex can inhibit the liberation of the proteolytic enzyme, a process which Macfarlane has recently suggested is the basis of the adaptation syndrome.

*Nature of the stimulus.* There is no doubt about the fundamental importance of the pituitary in the process of adaptation. It is, however, possible that noxious stimuli first act directly on the cortex, damage it, decrease the amount of circulating cortical hormones, and thus stimulate the production of corticotrophic hormone from the pituitary. This attractive hypothesis is supported by many facts, and is amenable to experimental test.

Whichever gland is first affected, the nature of the immediate stimulus is unknown. Uotila [1939] has shown that it cannot follow a nervous pathway. Among chemical stimuli, hypoglycaemia [Lewis *et al.* 1942] and alkalosis [Langley & Clarke, 1942] have been suspected. A most interesting suggestion is that an increase in protein breakdown products is the main factor responsible for adreno-cortical stimulation [Tepperman *et al.* 1943]. The blood non-protein nitrogen rises in almost all cases of alarm reaction, and this may well be the factor that starts the whole chain of events. This hypothesis can be linked with the view about proteolytic enzymes that has already been discussed. Thus it is not inconceivable that any stimulus that causes protein destruction gives rise, through the adrenal cortex, to a process controlling proteolysis.

These, however, are just speculations, and much hard work and clear thinking will be required before a satisfactory interpretation can be provided for the phenomena I have outlined.

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**Biological assays of cortical hormones and estimation of the rate of secretion of the mammalian suprarenal cortex.** By MARTHE VOGT. *Department of Pharmacology, University of Edinburgh*

I. *Biological assays*

Several procedures have been suggested for the assay of cortical hormones, a fact which, in itself, indicates that there is no completely satisfactory method. They fall into three groups:

(1) Maintenance or prolongation of life in adrenalectomized animals (dogs, cats, rats, mice, drakes).

(2) Tests on normal animals or tissues: augmented potassium excretion [West, 1942]; sodium retention [Hartman, Lewis & Thatcher, 1941]; oedema of perfused hindlimbs [Hyman & Chambers, 1943; not confirmed by Palmer & Joseph, 1946]; semi-contraction of melanophores of scales of the carp [Giroud, Santa & Martinet, 1939]; growth of ovipositor of bitterling [Duyvené de Wit, 1941].

(3) Tests which measure the improvement by cortical hormone of some deficiency which is produced by adrenalectomy:

(a) Growth [Grollman, 1941].

(b) Non-protein-nitrogen in dogs' blood [Piffner, Swingle & Vars, 1934].

(c) Performance of muscular work [Ingle, 1944; Everse & de Fremery, 1932].

(d) Water diuresis [Petrányi, 1941].

(e) Resistance to water intoxication [Eversole, Gaunt & Kendall, 1942].

(f) Resistance to histamine poisoning [Perla & Gottesman, 1931].

(g) Resistance to potassium poisoning [Feil & Dorfman, 1945].

(h) Resistance to shock [Elmadjien & Pincus, 1944].

(i) Resistance to typhoid vaccine [Lewis & Page, 1946].

(j) Glycogen deposition in liver [Reinecke & Kendall, 1942]; sensitive modifications [Eggleston, Johnston & Dobriner, 1946; Venning, Kazmin & Bell, 1946].

(k) Survival of exposure to low temperature [Selye & Schenker, 1938].

(l) Fall in body temperature on exposure to low temperature [Tyslowitz & Astwood, 1942].

Most of the information on the amounts of cortical hormones present in body fluids has been obtained with tests 3 (j) (glycogen deposition) and 3 (k) (exposure to low temperature). Test 3 (j) is peculiar in so far as responses are only obtained with corticoids having an oxygen atom attached to C<sub>11</sub>. In its newer modifications, it is sufficiently sensitive to be used for the assay of corticoids in urine. Test 3 (k) is a little more sensitive than 3 (j), and responses are obtained with all types of corticoids which maintain life in the adrenalectomized animal.

Whenever the accuracy of these biological assays has been calculated it has been found to be low. In test 3 (*k*), for instance, the results are subject to great individual and day-to-day variations, and the slopes of the dose-response curves are variable and not steep. The response plotted against the logarithm of the dose gives a straight line only over a narrow range. Significant results can only be obtained if the tests are carried out on litter mates of the same weight, a fact which impairs the practicability of the test. Similar difficulties are often encountered in vitamin tests, and the reason may be that the corticoids, like some vitamins, affect the metabolism of all tissues, each of which offers chances for variations in the response.

Another difficulty is the lack of a standard. It would seem reasonable to use one of the crystalline corticosterone derivatives for this purpose, but it is the experience of Olson, Jacobs, Richert, Thayer, Kopp & Wade [1944] as well as of the author, that regression lines for extracts and pure steroids are not parallel, and that their comparison is therefore not possible except at arbitrary levels.

## II. *Biological applications*

Canadian and American workers have used some of these methods for the estimation of cortical hormone in human urine. Whereas the first figures obtained were either too high or too low, several investigators have now established with a reasonable degree of certainty the normal figures for daily human excretion of corticoids in the urine, and also the higher values that are obtained after operations, burns, and exercise or in influenza, Cushing's disease and pregnancy. The normal figures found by Venning & Kazmin [1946] correspond to the activity of 40  $\mu$ g. Kendall E (contained in about 8 g. gland) for the female, and of 60  $\mu$ g. (contained in about 12 g. gland) in the male.

A certain amount of caution in the physiological interpretation of these data is, however, necessary, since the kidney only excretes a minute fraction of injected or (see below) secreted hormone.

A more direct approach to the problem of the secretory activity of the adrenal cortex was made by applying the method of Selye & Schenker [1938] to the assay of the hormone in the suprarenal blood of animals, and the following is a survey of the results of such experiments [Vogt, 1943, 1944].

Nearly undiluted venous blood from the adrenals of a dog can be obtained by cannulating, in a heparinized anaesthetized animal, the first lumbar vein of one side. Appropriate modifications of this technique may be used in animals in which the suprarenal effluent does not run into the first lumbar vein (pig, goat and rabbit). The Selye-Schenker test proved to be sufficiently sensitive to permit the determination of cortical hormone in this blood, whereas arterial blood or heart blood of the same animals never contained an amount of hormone that could be determined by the method. Figures for the concentration of cortical hormone found in adrenal blood were, in the absence of a better standard, expressed in terms of the activity of commercial extracts ('Eucortone', Allen and Hanbury) which, though varying in their properties, are always prepared by extraction of the same weight of slaughterhouse glands. The results of such comparisons, carried out on a large number of different mammals, are given in Table 1.

Table 1

Range Average	Rate of secretion of adrenal cortex in terms of		
	'g. slaughter-house gland'		Output per min. as multiple of quantity extractable from secreting gland
	per min. per kg.	per ml. adrenal plasma	
	0.26-2.0	2.1-10.0	5-29
	0.6	7.0	10

The average output of cortical hormone, per min. and per kg. body weight, is 0.6 'g. gland', a figure which would correspond to a production, by both glands of a 10 kg. dog, of 720 g./hr. or of 17 kg. gland/day. This last figure corresponds, as far as such comparisons can be trusted, to 54 mg. Kendall E, which is of the order of 1000 times the total daily excretion in human urine. It is interesting that similar figures were arrived at by Olson, Thayer & Kopp [1944] by a completely different approach. They determined the amount of hormone required by a suprarenalectomized rat to attain the fasting glycogen level of a normal rat, and concluded that an equivalent of 876  $\mu$ g. of corticosterone were secreted daily by the fasting normal rat. This value is in surprisingly good agreement with the figure of 0.6 g. gland/min./kg. which corresponds to 864  $\mu$ g. Kendall E/day/rat. Kendall E being somewhat more active than corticosterone, the equivalent corticosterone value would be a little larger.

The finding that very large quantities of glandular tissue would have to be extracted in order to obtain the equivalent of the daily output of the suprarenal of a single animal probably means that the hormone is not stored in the gland in its active form but is continually synthesized from an inactive precursor and immediately released into the circulation. The alternative explanation, that our present methods of extracting the hormone are inadequate, is unlikely, as it was found that large amounts of hormone were easily extracted from adrenal affluent. Further, the adrenals are known to have, per g. tissue, a larger blood flow than most organs, and it is conceivable that they require this flow in order to perform continually the synthetic processes for the elaboration of the cortical hormone.

The secreted hormone disappears from the blood at great speed and is obviously taken up by the tissues. Experiments, in which eviscerated and nephrectomized animals were tested for cortical hormone several hours after these operative procedures had been completed, showed no detectable accumulation of the hormone in the arterial blood. This shows that neither the liver nor the kidneys are essential for the uptake, destruction or excretion of the circulating hormone.

The next problem concerns factors which may affect the rate of cortical secretion. Whereas blood pressure and blood flow were found to be without effect, stimulation of the splanchnic nerves influenced the rate of secretion. Hence, since such stimulation releases adrenaline, the action of this substance had first to be investigated.

Intravenous infusions of small doses of adrenaline, such as would be secreted during a short stimulation of the splanchnic nerves (6-8  $\mu$ g./kg.) cause a rapid and large increase in the rate of cortical secretion which may last for a quarter to half an hour. If larger doses are given, the response is of longer duration. The secretion rates obtained are of the order of several times the basal level.



Since the nerves to the suprarenal apparently do not send any fibres to the cortex but supply only the medulla, this effect of adrenaline on the secretion of the cortex obviously represents the means by which the sympathetic system exerts an indirect nervous control over the secretory activity of a gland which has no innervation of its own. Stimulation of the splanchnic nerves while the venous effluent of the adrenals (which contains most of the liberated adrenaline) is not returned into the general circulation, has little or no effect on cortical secretion. Hence, it is likely that the effect of the splanchnic nerves on cortical output is entirely effected by the release of adrenaline. The purpose of this increased output seems to be to help the body to resist sudden stress or strain.

Finally, the Selye-Schenker test was applied to the examination of the secretion of the isolated perfused gland. Perfusion of dogs' glands was carried out with heparinized blood by means of a Dale-Schuster pump. In such experiments, it is possible to test the direct effect of the composition of the blood on the suprarenal tissue. Isolated glands were found to secrete continuously at a steady rate; the amounts secreted per minute lay within the limits observed in the experiments on the whole animal though on the average they were somewhat lower. Several metabolites (glucose, lactic acid, amino-acids) were added to the perfusing blood without having any effect on the secretory rate. Of the drugs tested (histamine, nicotine and potassium chloride), only histamine occasionally produced a transient rise in output. Large quantities of corticotrophic hormone, however, caused an immediate and prolonged increase in the rate of cortical secretion. These findings are in good agreement with the experience on the intact animal, in which the role of the pituitary in the control of cortical activity is known to be of pre-eminent importance.

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In summing up the symposium, the Chairman, Prof. ZUCKERMAN, pointed out the paradox that although extra-dietary NaCl will maintain an adrenalectomized animal in good health under controlled environmental conditions, the secretory activity of the adrenal glands is normally surprisingly high, and rapidly rises to even higher levels under the influence of environmental stimuli. The importance to the body of this secretory activity, and the fate of the secreted steroids, remain in the forefront of biological problems. Although the cortical secretion might not be essential for survival under some conditions, and did not necessarily initiate reactions in the body, but hastened or retarded enzymically controlled processes, the importance of adrenal

## ERRATUM

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Proceedings, page lxi

Some observations on the determination of pregnanediol.

By I. F. Sommerville, N. Gough and G. F. Marrian

2nd para. line 3. *For*

"Low and irregular recoveries were observed with slow cooling."

*Read*

"Low and irregular recoveries were observed when the hot mixture was cooled rapidly, whereas higher and more regular recoveries were obtained with slow cooling."

was proposed by Astwood & Jones [1941] and further developed by Talbot, Berman, MacLachlan & Wolfe [1941] has been studied with the object of elaborating a reliable method for the determination of the small amounts of pregnanediol in the urine of non-pregnant women.

The recovery of pure pregnanediol added to the toluene-soluble neutral fraction of acid-hydrolysed human male urine in the ethanol-N/10-NaOH or water hot precipitation process, which is an essential feature of the method, has been investigated. Low and irregular recoveries were observed with slow cooling. This difference was most marked with amounts of pregnanediol equivalent to less than about 2.5 mg./24 hr.

Experiments carried out on the neutral fraction of male urine without added pregnanediol have shown that a single precipitation process as used by Talbot *et al.*

Since the nerves to the suprarenal apparently do not send any fibres to the cortex but supply only the medulla, this effect of adrenaline on the secretion of the cortex obviously represents the means by which the sympathetic system exerts an indirect nervous control over the secretory activity of a gland which has no innervation of its own. Stimulation of the splanchnic nerves while the venous effluent of the adrenals (which contains most of the liberated adrenaline) is not returned into the general circulation, has little or no effect on cortical secretion. Hence, it is likely that the effect of the splanchnic nerves on cortical output is entirely effected by the release of adrenaline. The purpose of this increased output seems to be to help the body to resist sudden stress or strain.

Finally, the Selye-Schenker test was applied to the examination of the secretion of the isolated perfused gland. Perfusion of dogs' glands was carried out with heparinized blood by means of a Dale-Schuster pump. In such experiments, it is possible to test

35, 430.

- Olson, R. E., Thayer, S. A. & Kopp, L. J. [1944]. *Endocrinology*, **35**, 464.  
Palmer, G. H. & Joseph, G. H. [1946]. *Amer. J. Physiol.* **146**, 126.  
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Dr A. S. PARKES moved a vote of thanks, which was passed with acclamation, to the Dean and Faculty of Medicine of the University of Birmingham for arranging the meeting. Prof. A. P. THOMSON replied on behalf of the Medical School.

## SECOND ANNUAL GENERAL MEETING

*Held at University College, Gower St, London, on 29 May 1947*

**Some observations on the determination of pregnanediol.** By I. F. SOMMERVILLE, N. GOUGH and G. F. MARRIAN. *Department of Biochemistry, Edinburgh University*

The method for the quantitative determination of pregnanediol in human urine which was proposed by Astwood & Jones [1941] and further developed by Talbot, Berman, MacLachlan & Wolfe [1941] has been studied with the object of elaborating a reliable method for the determination of the small amounts of pregnanediol in the urine of non-pregnant women.

The recovery of pure pregnanediol added to the toluene-soluble neutral fraction of acid-hydrolysed human male urine in the ethanol-N/10-NaOH or water hot precipitation process, which is an essential feature of the method, has been investigated. Low and irregular recoveries were observed with slow cooling. This difference was most marked with amounts of pregnanediol equivalent to less than about 2.5 mg./24 hr.

Experiments carried out on the neutral fraction of male urine without added pregnanediol have shown that a single precipitation process as used by Talbot *et al.*

and by Guterman [1944] is much less efficient for the removal of  $H_2SO_4$  chromogens other than pregnanediol than is the original triple precipitation of Astwood & Jones.

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## Observations on the excretion of diethylstilboestrol by the ruminant. By F. H. MALPRESS and E. C. OWEN. *Department of Biochemistry, Queen's University, Belfast*

Folley & Malpress [1944] observed that diethylstilboestrol administered to young heifers was far less efficient in promoting lactation when given orally than when given by implantation or injection. In the preliminary study reported here an attempt was made to determine whether this was due to destruction of the oestrogen in the rumen or to its poor absorption from the intestine; some observations were also made on the possible nature of the conjugated excretory products of this oestrogen.

4.5 g. diethylstilboestrol were fed to a dry cow after a control period of 17 days; complete collections of the excreta were made throughout the experiment, and the free and ether-soluble conjugated urinary diethylstilboestrol fractions and the faecal diethylstilboestrol excretion measured [Malpress]. Oestrogen was found in the urine, both in the free form and in the ether-soluble (glycuronide) form during the 5 days immediately following feeding, while faecal excretion continued for a further 6 days. The determined absorption and recovery data are given in Table 1.

Table 1

	mg.	Recovery (%)
Diethylstilboestrol ingested	4500	—
Diethylstilboestrol faecal excretion	1043	23.2
Diethylstilboestrol urinary excretion:		
(i) free	252	5.6
(ii) conjugated	277	6.2

The faecal values were obtained by a method based on the recovery of diethylstilboestrol added in ether solution to dry, ground faecal samples. It became clear during the analysis of the experimental samples, however, that this *in vitro* treatment did not satisfactorily reproduce the much stronger state of adsorption on to faecal particles of the unabsorbed ingested oestrogen; the faecal recovery of 23.2 % must be regarded therefore as a minimum figure. The extent of rumen destruction, if any, consequently still remains in doubt, but it is clear that the absorption of unchanged oestrogen is by no means complete, a fact borne out by the low urinary excretion.

In a second experiment a dose of 121 mg. diethylstilboestrol in olive oil was injected subcutaneously into a dry cow; no rise in the excretion of conjugated ether-soluble urinary diethylstilboestrol was found and the glycuronic acid excretion was also unaffected. The ethereal sulphate excretion and ratio of ethereal:inorganic sulphate

Table 2

Period (3-day)	Inorganic S (mg./day)	Ethereal S (mg./day)	E:I	Glycuronic acid (g./day)
Pre-injection I	3.80	2.97	0.80	6.9
Pre-injection II	6.20	4.80	0.80	8.8
Post-injection I	6.31	7.20	1.13	5.6
Post-injection II	5.43	4.38	0.82	5.7
Post-injection III	3.98	3.25	0.82	2.3

All figures represent mean values over 3-day periods.

excretion, however, were both definitely increased (Table 2). After oral administration of oestrogen in the first study recorded here the latter index only was raised. These changes suggest that, to some extent, diethylstilboestrol is detoxicated in the ruminant by combination with sulphuric acid. This hypothesis, however, requires further investigation.

We wish to thank Dr R. T. Williams of Liverpool University for a sample of recrystallized *d*-glucurone used in this work.

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**The antithyroid activity of ergothioneine.** By A. LAWSON and C. RIMINGTON.  
*Department of Chemical Pathology, University College, London, W.C. 1*

The ergothioneine used was extracted from ergot of rye. It was administered in graded doses subcutaneously or by mouth to young rats (50 g.) over a 10-day test period. For purposes of comparison the same dosages of thiouracil were given to a corresponding number of animals. The antithyroid potency assay was based on the diminution of the iodine content of the thyroid glands of the animals, determinations being made on each pair of glands.

Ergothioneine administered by both routes in dosages up to 5 mg./kg. body weight had an antithyroid activity very close to that of thiouracil, and was twice to three times more active subcutaneously than by mouth.

Two points of interest arise from the finding that ergothioneine has antithyroid activity. The first is that this substance is a normal constituent of mammalian blood, being present according to other workers to the extent of 3–10 mg./100 ml. of human corpuscles. The second point of interest is that, if the concentration levels of blood ergothioneine, corresponding to the dosage levels at which marked antithyroid activity is observed, are estimated from data of dosage and calculated blood volume, values result which are of the same order as those known to obtain normally. Whether significant variations of blood ergothioneine occur in persons with disturbance of thyroid function is unknown.

**Further observations on the closed vessel technique for testing thyroid activity.** By AUDREY U. SMITH. *National Institute for Medical Research, London, N.W. 3*

The survival time (s.t.) of mice in closed vessels was prolonged by treatment with thiourea. Effective doses by subcutaneous injection were 0.1 and 0.4 mg. daily for 5 or 10 days, and 0.025 mg. daily for 10 or 20 days. Administered orally, concentrations of 0.01, 0.04 and 0.16 % in the drinking water for 5 or 10 days were effective. Large doses of thiourea by injection or orally for 20 days caused a high mortality and morbidity rate during the period of treatment, and also shortened the s.t. of the mice in closed vessels.

A single subcutaneous injection of 0.005 or 0.025 mg. of racemic thyroxine shortened the s.t. of mice when tested 24, 48 or 72 hr. after the injection. *l*-Thyroxine was considerably more active than *d*, *l*-thyroxine in reducing the s.t. of mice, and *d*-thyroxine was almost inactive.

A single injection of di-iodothyronine (0.05–0.4 mg.) reduced the s.t., doses ten times as large as those of *d*, *l*-thyroxine being required to produce comparable effects.

The following substances in the range of doses given were ineffective:

Thyroxamine (0.05–0.2 mg.).  
Di-iodothyronamine (0.05–0.2 mg.).  
Tetrachlorothyronine (0.05–2.0 mg.).  
Tetrabromothyronine (0.05–1.0 mg.).  
Di-iodotyrosine (0.5–20.0 mg.).

**Progesterone secretion during the oestrous cycle of the unmated rat.** By P. CONSTANTINIDES. *Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1*

Hooker's [1945] observations on the effects of oestrogen and progesterone on the endometrium of the spayed mouse have been confirmed, with minor differences, in the spayed albino rat.

Oestradiol (3 doses of 30  $\mu$ g. on 3 alternate days) does not produce as much stromal oedema as it does in the uterus of the spayed mouse but it causes more granulocytic infiltration [cf. Allen, 1931].

Progesterone (three daily doses of 0.5–3 mg.) enlarges the stromal nuclei, particularly those in the zone near the epithelium. These enlarged nuclei have their chromatin packed at the nuclear membrane and possess a large single or double nucleolus. There is some correlation between the daily dose of progesterone and the mean diameter of the stromal nuclei, and this is being investigated in more detail.

The fibroblastic character of the changes produced by progesterone in the nuclei of the stromal fibrocytes led to an investigation of the mitogenic aspect of this reaction. 9½ hr. after a colchicine injection the uterus of the spayed rat (3 weeks after ovariectomy) had only 0–6 mitoses per cross-section; after three daily doses of 3 mg. progesterone and colchicine this number was increased to 700–1000. Most of these mitoses were in the stroma, particularly in the zone immediately under the epithelium;

a few were scattered in the myometrium; none was found in the epithelium or the glands [Mussio Fournier, Albrieux & Buno, 1940].

Half this dose of progesterone produced 30–70 mitoses per cross-section, more dispersed throughout the stroma. Three injections of 30  $\mu$ g. oestradiol given during 3 alternate days together with the lower dose of progesterone did not apparently affect the stromal mitoses but produced in addition 10–30 mitoses per cross-section in the epithelium and glands.

Examination of the stromal nuclei of uteri from unmated rats killed at intervals during regular 4-day oestrous cycles suggested that progesterone (or a progesterone-like substance) was active throughout the cycle, though not uniformly. The effect was minimal during early metoestrus and maximal during late pro-oestrus. During early metoestrus the nuclei of the stromal cells just below the epithelium were nearly all in the pycnotic fibrocytic state [cf. Nishida, 1936]. The recovery of their fibroblastic appearance began during the first day of dioestrus and was complete by the second day.

These observations support Long & Evans's [1922] histological evidence that the corpus luteum of the normal cycle reaches its greatest development at the end of the cycle in which it is formed, and with Boling's [1942] findings that it rapidly regresses during the beginning of the next cycle.

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# PROCEEDINGS OF THE SOCIETY FOR ENDOCRINOLOGY

## SYMPOSIUM ON THE ASSAY OF URINARY STEROIDS

*Held at Guy's Hospital Medical School, London, S.E. 1 on 28 June 1947*

**The chemical estimation of urinary neutral 17-ketosteroids.** By R. K. CALLOW.  
*National Institute for Medical Research, London, N.W. 3*

The period which has elapsed since chemical methods of estimating 17-ketosteroids were first elaborated allows the subject to be viewed in perspective, and the current revival of interest makes this a most suitable occasion for a review.

Zimmermann [1935, 1936] first applied the colour reaction given by methylene-ketones, i.e. compounds containing the group  $-\text{CH}_2\text{CO}-$ , with *m*-dinitrobenzene and alkali to the determination of 17-ketosteroids. He successively added to 2 parts of an absolute alcoholic solution of the test substance, 1 part of a 1% solution of *m*-dinitrobenzene in absolute alcohol and 1 part of aqueous 3*N*-KOH, and measured the colour after 60 min. He pointed out that several important compounds with sex-hormone activity contained this  $-\text{CH}_2\text{CO}-$  group, either in the 2:3- or the 16:17-positions. 17-Keto-compounds were found to give characteristic colours, with characteristic absorption spectra.

A portion of Zimmermann's 1936 paper is devoted to a preliminary study of the application of the reaction to urine extracts. He concluded that the absorption spectra of these extracts corresponded to mixtures of 95% 17-ketone and 5% creatinine, and noted that the 17-keto-compounds determined chemically and calculated as androsterone exceeded the amount to be expected from the biological activity.

The further study and application of this reaction was taken up by several other workers, Wu & Chou [1937] in China, Oesting [1937] in America, and by Callow, Callow & Emmens [1938]. The work undertaken by ourselves had three successive objectives: (1) to increase the sensitivity and reproducibility of the reaction, (2) to study the application of the method to urine extracts, and (3) to correlate the results of chemical estimation with those of biological estimation of androgens in urine. These aspects will be briefly dealt with in turn.

Increased sensitivity was attained by varying the conditions of the reaction, e.g. concentration of reagents, so as to obtain the maximum intensity of colour. At the same time reproducibility of the reaction was increased as far as possible, by setting a standard of purity for the reagents, and by choosing conditions for the reaction such that minor variations would have little effect, i.e. the intensity of colour should be at a maximum, but preferably at a *flat* maximum.

So many factors were concerned that the investigation was never complete, and it is not surprising that subsequent workers have introduced their own modifications. Perhaps the most noteworthy of recent work is that of Zimmermann himself, who has published [Zimmermann, 1944*a, b, c, d, e, f*] a very detailed study culminating in recommendations for using aqueous alkali, which he considers a most rapid and most convenient method for routine use. Table 1 shows, for comparative purposes, the 1936 Zimmermann, the 1938 Callow, Callow & Emmens, and the 1944 Zimmermann methods.

Table 1. *Comparison of methods for colorimetric estimation of 17-ketosteroids*

	Zimmermann [1936]	Callow, Callow & Emmens [1938]	Zimmermann [1944]
Substance for test in abs. ethanol	2 ml.	0.2 ml.	1 ml.
m-Dinitrobenzene in abs. ethanol	1 %; 1 ml.	2 %; 0.2 ml.	2 %; 1 ml.
KOH	3N; 1 ml. (in H <sub>2</sub> O)	2.5N; 0.2 ml. (in EtOH)	3N; 1 ml. (in H <sub>2</sub> O)

In each case colour was developed for 60 min. at constant temperature and protected from light. Zimmermann measured the colour undiluted, while Callow, Callow & Emmens added 10 ml. EtOH before measurement.

A variation of some interest is provided by the possibility of extracting the colour from the reaction-mixture, either by ether, as was done by Zimmermann [1944*b*] or by chloroform, as was done by Cahen & Salter [1944]. Zimmermann found his method poorly reproducible, but Cahen & Salter concluded that chloroform extraction was advantageous because it isolated the 'desirable pink colour' and left unspecific colours in the aqueous-alcoholic layer.

The degree of specificity of the Zimmermann reaction is an important point. It was shown early on that within the steroid group the colour with a sharp maximum of absorption in the green was peculiar to 17-ketosteroids. Other substances, e.g. acetophenone, may simulate 17-ketosteroids, but no such non-steroid compound has, so far, been recognized as causing interference in urine extracts suitably fractionated by the simple methods in use. Simultaneous presence of 3- and 20-ketosteroids will, however, affect the absorption spectrum by adding general absorption, and Zimmermann [1944*d*], for example, has considered the possibility of the simultaneous determination of androsterone and testosterone in mixtures.

Excellent as these methods may be for the determination of pure 17-ketosteroids, the transference of this model-research to urine raises numerous difficulties. It is well known that steroid derivatives are excreted in the urine in conjugated, water-soluble forms. The liberation of the steroids is necessary for either biological or chemical determination. One method in general use is hydrolysis by boiling with 1.5N-hydrochloric acid, with either simultaneous or subsequent extraction by an immiscible solvent. The isolation of artefacts such as chloroandrostenone [Butenandt & Dannenbaum, 1934] and androstenone [Venning, Hoffman & Browne, 1942] derived from androsterone indicate, however, that decompositions may occur. Talbot, Ryan & Wolfe [1943] elaborated a method—not generally accepted—of hydrolysis by barium chloride, which is claimed to minimize decomposition.

Fractionation of urine extracts as a preliminary to colorimetry constitutes a problem which is bound up with that of dealing with chromogenic impurities. The highly coloured, crude extract of hydrolysed urine is obviously unsuitable for direct colorimetry, and phenols and acids, together with much coloured material, are removed by alkali. The colour reaction with the extract purified to this stage gives absorption spectra which vary in the degree of selectivity of absorption. While some extracts give 'clean' colours, the 17-ketosteroid curve with its maximum in the green standing out boldly, others give colours with notable absorption in the violet, and numerical comparison of the absorption coefficients in the green is obviously misleading.

Callow, *et al.* [1938] took the easy course of simply neglecting such results; where the values of the ratio  $E_v/E_g$  exceeded 0.8. Urines giving colours of this type had low contents of 17-ketosteroids, and were, from their point of view at that time, of little interest. Fraser and his co-workers [Fraser, Forbes, Albright, Sulkowitch & Reifstein, 1941], Talbot and his co-workers [Talbot, Berman & MacLachlan, 1942] and Engstrom & Mason [1943] applied correction factors, derived from the absorption in the violet, to give the value due to 17-ketosteroids free from contaminants with unselective absorption. Oesting & Webster [1938] cleaned the extracts before colorimetry by treatment with active charcoal, but several workers found that 17-ketosteroids were removed by this method. Using another line of approach, Talbot, Butler & MacLachlan [1940] developed the separation of the ketonic from the non-ketonic neutral substances by means of Girard's reagent and so removed a portion of interfering substances. This type of work reached a peak with a method of fractionation [Pincus & Pearlman, 1941] in which acids, phenols, non-ketonic compounds, non-hydroxylic compounds, and hydroxylic  $\alpha$ - and  $\beta$ -steroids were separated successively. Excellent as this scheme may be, it is obviously impractical for routine investigation, and is only applicable to research problems.

Much might be said in favour of the view that chromogenic impurities are of secondary importance except in special cases. Excluding this limited class, there is serious interference with determinations only when the quantity of total 17-ketosteroids is low, but when the quantity is low the exact figure is unimportant for most purposes. When the total of 17-ketosteroids is high, refinements of the analysis, leading to further differentiation of the excreted steroids, become important. Attempts to separate the so-called  $\alpha$ - and  $\beta$ -steroids, the 3( $\alpha$ )- and 3( $\beta$ )-hydroxy-compounds typified by androsterone and aetiocholanolone on the one hand and dehydroandrosterone on the other fall under this heading, while Baumann & Metzger [1940] and Frame [1944] have elaborated methods of separation by treatment with digitonin, which precipitates the  $\beta$ -hydroxy-compounds.

De Laat [1941] investigated the chromatographic separation of androsterone and dehydroandrosterone on a small scale, and this was developed later [Dingemans, Huis in't Veld & de Laat, 1946] into a promising method by which extracts from small samples of urine were eluted from an adsorption column, and the results expressed graphically in such a way that an elution-pattern of 17-ketosteroids was obtained with peaks corresponding to a number of different compounds. Qualitative and roughly quantitative comparisons of normal and abnormal urines can be made from the graphs.

A simple routine method of estimating the relative amounts of different hydroxy-steroids can be developed by applying the Pincus method [Pincus, 1943] of colorimetric determination, which depends on the development of a typical colour, with an absorption maximum at 630  $m\mu$ ., when a dry preparation reacts at 80–100° with a solution of antimony trichloride in acetic acid with some acetic anhydride. The colour is given by 3-hydroxy-steroids, whether  $\alpha$  or  $\beta$ , saturated in ring I, but not by dehydroandrosterone. Salter, Cahen & Sappington [1946] suggested that determinations on the same urine of the total 17-ketosteroids by the Zimmermann method, and of the 17-ketosteroids reacting with the Pincus reagent may be of diagnostic value.

In the early days of this work attempts were made to correlate—it might even be said, to reconcile—chemical determinations and biological assays on urine extracts. A compelling reason at the time for attempting such a correlation was the necessity of defending a new method against the old. The biological assayers argued plausibly that the androgenic activity of the urine was a mirror of the androgenic activity of the blood. In default of the detailed chemical knowledge we now have of the metabolism of androgens this argument had to be accepted and chemical results reconciled with biological results. The position is now different, and chemical methods can stand on their own. The arguments for the chemical method—or equally for the physico-chemical method of polarography—are first, ease, cheapness, rapidity and reproducibility, and second, that, so far as we do know anything about metabolism of androgens in the body, the process is with few exceptions one of conversion into 17-ketones. It must be emphasized, however, that there is very little information about the conversion of adrenocortical compounds into 17-ketosteroids.

The origin in the body of excreted 17-ketosteroids rests on a purely empirical basis. We are far from supposing that urinary excretion directly reflects circulatory conditions. The position is, rather, that the 17-ketosteroids in urine form a fraction which can be grossly estimated and analysed into components, and the research going on now consists of attempts to correlate the results with clinical and pathological observations.

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The ensuing discussion centred on the difficulty of getting satisfactory colour correction formulae. The corrections are more variable than the single formula of Talbot suggests. It seems that the nature of the alcohol used in the reaction affects the colour, and that the green filters may also vary in their light transmission.

**Comparison between polarographic and other chemical methods for the determination of urinary neutral 17-ketosteroids.** By C. J. O. R. MORRIS.  
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In addition to the various colorimetric methods for the estimation of neutral 17-ketosteroids in urine, a polarographic method can also be used for the same purpose. The method depends on the cathodic wave given by the hydrazones of ketonic steroids with the Girard T. reagent. It is possible by the polarographic technique to distinguish 3- from 17-ketosteroids, and further to estimate both simultaneously in the same extract. The principle has now been developed into a routine method of high reproducibility for the estimation of neutral 17-ketosteroids in urine [Barnett, Henly & Morris, 1946].

With the co-operation of Dr F. L. Warren of the Physiology Department, St Mary's Hospital, and Mrs A. M. Robinson of the Pathology Department, St Bartholomew's Hospital, a comparison has been made between the values obtained on the same extracts by the polarographic and Zimmermann colorimetric methods. Both normal and pathological cases were examined. A preliminary study [Barnett, Henly, Morris & Warren, 1946] of eight normal males and twenty normal females indicated that the colorimetric method gave consistently higher values than the polarographic method, and that the reproducibility of the latter was better.

A further comparison in nineteen cases has now been made between polarographic values and Zimmermann colorimetric values corrected by the method of Talbot, Berman & MacLachlan [1942]. The results show that the corrected colorimetric values are in much better agreement with the polarographic values than the uncorrected estimations, so that the use of the colour correction is of definite value.

There are, however, certain extracts where a large discrepancy still remains, and it appears that, at least in pathological cases, there are chromogenic substances present which are not measured as 3- or 17-ketosteroids by the polarographic method.

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**Chemical assay of urinary oestrogens.** By G. F. MARRIAN.  
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#### INTRODUCTION

The only important chemical methods for the assay of urinary oestrogens are those based on the well-known Kober [1931] reaction. This reaction consists of heating the oestrogen with a mixture of phenol and concentrated sulphuric acid, diluting with water and reheating. A pink colour solution having an absorption maximum at 520 m $\mu$  is obtained. The reaction is highly specific to the natural oestrogens, and when carried out under certain conditions pink colours of equivalent intensities are developed by oestriol, oestrone and  $\alpha$ -oestradiol. Improvements in the technique of

carrying out the colour reaction have been made by Cohen & Marrian [1934], Venning, Evelyn, Harkness & Browne [1937] and Bachman [1939*a*], amongst others. Various modifications in the composition of the reagent have also been made, the most important being the substitution of  $\beta$ -naphthol for phenol [Kober, 1938].

Specific colour reactions based on the Kober reaction have been proposed by David [1934] and by Bachman [1939*b*] for oestriol, and by Szego & Samuels [1940, 1943] for oestrone.

The Kober reaction may be used for the determination of the oestrogens in partially purified extracts of human and equine urine, but it may give very inaccurate results in certain circumstances and its use for this purpose is subject to certain limitations.

#### HYDROLYSIS OF CONJUGATED OESTROGENS AND EXTRACTION

As far as is known at present, oestrogens occur in urine as glucuronides and sulphates. Before they can be readily extracted with water-immiscible solvents these conjugated oestrogens must therefore be hydrolysed, the usual method being that of boiling the urine with acid. One of the most widely employed techniques is that of Smith & Smith [1935] in which the urine is boiled for 10 min. with 15 % by volume of concentrated HCl. As shown by Smith, Smith & Pincus [1938], this procedure is as efficient as the less convenient method proposed by Cohen & Marrian in 1935.

Recent investigations of the method [Stevenson & Marrian, 1947*a*] show, however, that boiling for 40–60 min. with 15 % concentrated HCl, instead of for 10 min., about doubles the amount of ether extractable 'Kober chromogen' in human pregnancy urine, while the addition of zinc increases the yield a further 10–30 %.

Ether appears to be the least unsatisfactory solvent for the extraction of oestrogens from acid-hydrolysed urine. Benzene has been widely used, but is relatively ineffective for the extraction of oestriol. Bachman & Pettit [1941] have, however, shown that the efficiency of benzene as an extraction solvent for oestriol can be greatly increased by saturating the acid-hydrolysed urine with HCl.

#### PURIFICATION OF OESTROGEN FRACTIONS

The total oestrogen in a urinary ether extract may be determined by applying the Kober reaction to the separated total phenolic fraction, which may be obtained by the following steps: (i) washing the ether extract with aqueous  $\text{NaHCO}_3$  to remove acidic substances; (ii) evaporating the washed ether extract and dispersing the residue in benzene with the aid of a small volume of ethanol; (iii) extracting the benzene solution so obtained with aqueous NaOH; and (iv) extracting the NaOH extract with ether after acidification.

As shown by Mather [1940, 1942], and independently by Bachman & Pettit [1941], oestriol can be readily extracted from benzene solution by aqueous  $\text{Na}_2\text{CO}_3$ , whereas oestrone and oestradiol cannot be so extracted. It is thus possible to separate the total phenolic fraction of urine into two fractions, one containing the oestriol and the other the oestrone and oestradiol. The total oestrogen in the latter fraction may be determined by the Kober reaction and the amount of oestrone by the Zimmermann method. One can therefore estimate separately the oestriol, oestrone and oestradiol in a urinary extract.

## BROWN COLOUR CORRECTION

Urinary oestrogen-concentrates usually contain substances which yield a brown colour in the Kober reaction. This brown colour shows considerable light absorption at  $520\text{ m}\mu$ , so that its presence in the final Kober reaction mixture results in an overestimation of the oestrogen actually present. With human urines collected during mid- and late pregnancy and containing more than about 10 mg. oestrogen/24 hr., the ratio of oestrogen pink to non-oestrogen brown colour is so high that the error is negligibly small. With urines containing less oestrogen, however, the overestimation caused by this brown colour may become considerable.

The attempts that have been made to minimize this brown colour error can be classified under two main headings:

(i) Attempts to measure the brown colour and then to correct for it.

(ii) Attempts to eliminate without loss of oestrogen the substances that give rise to the brown colour.

Cohen & Marrian in 1934 attempted to correct for the brown colour by measuring the pink in the reaction mixture before and after treatment with hydrogen peroxide—a treatment which as shown by Kober [1931] rapidly discharges the oestrogen pink colour. This method was unsatisfactory since some fading of the non-oestrogen brown colour resulted from the peroxide treatment.

A better method was devised by Venning *et al.* in 1937. They showed that the brown colour absorbs strongly at  $420\text{ m}\mu$  while the oestrogen pink is nearly transparent at this wave-length. Accordingly, by measuring the absorption at  $420\text{ m}\mu$  and by assuming that the spectrophotometric characteristic of the brown colour was constant from urine to urine, they were able to calculate how much of the observed absorption at  $520\text{ m}\mu$  was due to the brown colour. According to Bachman & Pettit [1941], however, it cannot be safely assumed that the brown colours from different human urine specimens are spectrophotometrically identical.

Jayle, Crépy & Judas [1943] made use of the instability of the oestrogen pink colour in the presence of acetone for a method of brown colour correction. They used the  $\beta$ -naphthol reagent, and the reaction was carried out in the presence of acetone as recommended by Kober [1938]. The colour was measured spectrophotometrically immediately after development and again after several hours, the difference giving them the corrected absorption due to the oestrogen pink colour.

A rather similar method has been worked out independently by Stevenson & Marrian [1947*b*]. It has been found that whereas the oestrogen pink is completely discharged by heating at  $100^\circ$  for  $1\frac{1}{2}$  hr., the brown colour given by the total phenolic fraction of human male urine is almost unaffected by this treatment. In the determination of total oestrogen in pregnancy urine, therefore, the absorption at  $520\text{ m}\mu$  is measured before and after heating, the difference giving the corrected absorption at this wave-length due to the oestrogen pink.

The value of this method of brown colour correction in the determination of total urinary oestrogen has been tested in a series of experiments in which oestriol and/or oestrone was added in varying amounts to acid-hydrolysed human male urine.



Table 1. *Recovery of oestrogen added to acid-hydrolysed male urine*

Total oestrogen added as mg. oestriol/24 hr. urine	Amount of phenolic fraction used as % of 24 hr. sample	% recovery of oestrogen	
		Direct Kober	Corrected Kober
2.13	1.0	185	104
2.30	1.0	172	94
4.40	1.0	130	104
5.30	1.0	123	95
7.25	0.4	129	112
12.8	0.4	98	89
16.5	0.4	104	102
36.0	0.1	88	89

The results clearly show the value of this simple method of brown colour correction when less than c. 10 mg. oestrogen/24 hr. are present in the urine. Unfortunately, however, the method does not appear to be of much value with urines containing less than about 2 mg. oestrogen/24 hr.

The necessity for a brown colour correction has been avoided by Bachman & Pettit [1941] in their method, in which oestriol and oestrone + oestradiol are separately determined. The oestriol fraction, obtained by extraction of a benzene solution with aqueous  $\text{Na}_2\text{CO}_3$ , was found to give little non-oestrogen brown colour in the Kober reaction—a finding which has been confirmed by Clayton & Marrian (1947)—while it was found possible to remove impurities giving rise to brown colour from the oestrone and oestradiol fraction by washing a benzene solution with moderately diluted  $\text{H}_2\text{SO}_4$ .

Stimmel [1946] has described a chromatographic ( $\text{Al}_2\text{O}_3$ ) method for the separation of oestriol, oestrone, and oestradiol fractions from human pregnancy urine. The three fractions so obtained were stated to give little brown colour in the Kober reaction as judged by absorption at 420  $\text{m}\mu$ .

#### LIMITATIONS OF THE KOBER METHOD

It is doubtful whether any of the published methods of correcting for or eliminating the brown colour produced by non-oestrogenic substances are reliable when less than about 2 mg. oestrogen/24 hr. are present in the urine. Since biological assays indicate that the maximum oestrogen excretion during the menstrual cycle is of the order of 0.1 mg./24 hr., the value of these colorimetric methods for the determination of oestrogen in the urine of non-pregnant women is somewhat questionable. However, Jayle, Crépy, Vandal & Judas [1946] have recently applied their method to the daily determination of oestrogen excretion throughout the menstrual cycle, and, although their figures may not have much quantitative significance, it is of very great interest that their excretion curves closely resemble in shape those obtained by other workers using biological methods of assay. These results suggest that further work directed towards improvement of methods might in the future lead to a reliable colorimetric procedure for the determination of oestrogen in the urine of non-pregnant women.

In conclusion it may be mentioned that Stevenson & Marrian [1947a] have recently found that the phenolic fraction of human male urine contains non-chromogenic material that considerably enhances the pink colour given by oestriol or oestrone in the Kober (phenol) reaction. This hitherto unobserved possible cause of error in the colorimetric determination of oestrogens in human pregnancy urine is being further investigated.

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In answer to questions concerning processes of hydrolysis, Prof. MARRIAN said that the most effective method was to allow decomposition of the urine for 10 days. He did not think that zinc hydrolysis caused the activation of inactive compounds.

## The biological assay of urinary oestrogens and androgens. By C. W. EMMENS. *National Institute for Medical Research, London, N.W. 3*

The urine of normal men and of non-pregnant women contains only a few  $\mu\text{g}$ . of oestrogen and a few mg. of androgen per litre or per day's output. It is primarily for this reason that urinary oestrogens are still assayed biologically, whereas the estimation of 17-ketosteroids by the colorimetric method has largely replaced the biological assay of androgens. In view of the importance of individual 24 hr. samples, particularly from women, and of the small amounts of material they usually contain, any biological test must be highly sensitive. Specificity is not a particular trouble with assays of either oestrogens or androgens, but consistent results are not usually obtained by workers using the same technique in different laboratories nor by the same investigator using different techniques at different times. While this lack of consistency is in part bound up with the degree of purity of extracts, it still occurs when crystalline compounds are compared. An insignificant inherent error, ease of working, rapidity, and low cost are additional factors which are desirable in assay-methods, but they are never combined in any one method.

# OESTROGENS

(i) *Allen-Doisy tests.* Before examining in any detail the assay of urinary oestrogens, it is instructive to review the position in relation to pure substances. It became apparent early on that the relative potencies of crystalline oestrogens depend on the particular method of comparison used. Thus, in tests using ovariectomized rats or

mice, the results in the same laboratory are influenced, among other things, by the nature of the solvent, the site of application and the number of treatments given (Emmens [1939] and Pedersen-Bjergaard [1939]).

Some of the discrepancy between results obtained by similar methods in different laboratories may be due to the use of impure or incompletely characterized compounds, but this would not account for discrepancies within the same laboratory when small modifications in technique are introduced. These are due to differing rates of absorption, and perhaps of metabolism and excretion, under different test conditions.

A crude urine extract contains a mixture of oestrogens and other substances. The potency it exhibits in any particular test will depend on the nature of this mixture and the test-method employed. Since no one at present can isolate the natural oestrogens in quantitative amounts from small samples of urine, the problem of dealing with impure mixtures of oestrogens remains. Clearly the most useful solution would be to find a test-method which does not differentiate between the various oestrogens concerned and is not affected by the presence of the other substances in the extracts. The assay would then give an estimate of the total weight of oestrogen present, irrespective of its nature, and would resemble the determination of 17-ketosteroids as an index of endogenous androgen production, or the Kober reaction in giving an index of endogenous oestrogen production.

The conditions under which the potencies of the various natural oestrogens most closely approach one another when injected are those of slow, but not too slow, absorption. This may be achieved by multiple injections in oil solution, by the addition of such a substance as palmitic acid, or by esterification. The presence of augmenting substances in impure extracts may so equalize the potencies of the oestrogens present that even a technique using two oil injections may give useful and reasonably consistent results. If impure extracts have to be assayed it may therefore be better to have them really impure. On the other hand, this implies a standardization of the technique of extraction which, from urine to urine, might nevertheless result in differing proportions of augmenting substances in the extracts. As a routine, this might be overcome by adding an excess of such substances. It is even doubtful whether, when dealing with urines not containing relatively vast quantities of androgen, it is worth while separating the androgens from the oestrogens. The inhibitory effect of androgens on oestrogens—which might be expected from the results of tests with pure compounds—is not apparent in such extracts. An extract containing, say, 10  $\mu$ g. of oestrogen and 10 mg. of androgen, would not be expected to exhibit interaction between the two, as the ratio of androgen to oestrogen in a vaginal smear test must be about 5000 or more to 1 for detectable inhibition to occur [Emmens & Bradshaw, 1939].

When mice are used, the Allen-Doisy test for vaginal cornification has the advantage of high sensitivity, so that the oestrogenic activity of a 24 hr. sample of male urine can be estimated with fair precision, and of normal female urine with considerable precision—'precision' meaning that whatever the true answer may be with the particular technique used, it is determinable within narrow limits of error—with a standard error of, perhaps,  $\pm 10\%$ .

A more recent modification of the Allen-Doisy test uses intravaginal application. The rat responds to small amounts of oestrogen in oil solution when introduced

directly into the vaginal canal; the mouse responds poorly to such preparations. Both species, however, respond very well and are very sensitive to oestrogens in aqueous or aqueous-glycerol solutions. The results of Mühlbock [1940] and Emmens [1940-41], who have used this modification with crystalline oestrogens in the mouse, are in excellent agreement. When two or three applications are made, the oestrogens are strikingly similar in potency, oestrone being the most active. The results of Mühlbock do not suggest any great difference in the dose-response lines for the three compounds, but those of Emmens indicate significant differences, which are large enough to make detailed comparisons of potency rather meaningless. It is to be hoped that this latter finding is not typical, because the intravaginal method offers the best hope of finding an assay method which differentiates little between the different oestrogens and which is not affected by other substances, or even by esterification [Emmens, 1940-41]. It has also been shown [Emmens, unpublished] that the presence of considerable amounts of androgen—relatively much more than could be expected in urine extracts—does not affect results. This 2- or 3-application intravaginal method seems to allow of the fairly complete local utilization of oestrogen, whether esterified or not, and the potency of the various highly effective oestrogens (including some of the synthetic compounds) is probably the same under such conditions. It would be well worth while to explore the method further and to discover whether a complete levelling of potencies is attainable.

Using an application of 0.01 ml., a concentration of about  $0.015\mu\text{g./ml.}$  is needed to administer the median effective dose of oestrone to a mouse. Since the urine of a normal woman contains about  $0.005\text{--}0.025\mu\text{g./ml.}$  of oestrogen, the concentration in 'neat' urine is at least on the borderline of effectiveness. Assay with 'neat' or slightly concentrated urine might therefore be feasible. Stadler & Lyons [1938] used two applications of 0.01 ml. of a urinary sludge in distilled water in the rat, giving total doses of 0.05-1.0 ml. of original urine, and obtained positive responses. They assayed urinary output as equivalent to from 15 to  $170\mu\text{g.}$  of oestrone per day.

The same considerations hold with the assay of whole blood. Albrieux [1941*a*, *b*], and after him Krichesky & Glass [1947], investigated the assay of blood oestrogens by the insertion of pellets of dried blood into the rat vagina, and obtained comparable results. They find that about 30 mg. of dried blood from women, or about 0.15 ml. of blood, gives positive responses in rats. Thus, 0.015 ml. of blood should suffice for a mouse, which in general requires about a tenth of the rat dose. This work suggests that it may soon be possible to make estimations of blood hormone concentrations using small quantities of untreated blood. Albrieux found that there is more oestrogen in the corpuscles than in the plasma, which suggests that whole blood should be used.

(ii) *Other test methods.* A variety of alternative, but not extensively investigated, tests for oestrogenic activity have been suggested. The Astwood [1938] and other tests [Lauson, Heller, Golden & Severinghaus, 1939; Evans, Varney & Koch, 1941], using the immature rat uterus, depend on a weight increase after the injection of oestrogen, and are about as sensitive as the usual Allen-Doisy test. The Astwood test is of interest because of its speed; it also seems to be fairly accurate, but no doubt suffers from all the drawbacks discussed earlier when dealing with injected oestrogens. The rapid weight increase in this test is due to the early accumulation of water in the uterus, which must be weighed fresh. The test of Hartman, Littrell & Tom [1946],

which depends on the opening of the vagina of immature or castrate rats or guinea-pigs, is of the most interest. This is again a local test—oestrogen in 0.02 ml. of solvent is injected on each side of the future site of vaginal opening. If the reaction is positive, the vagina opens within 10–96 hr. The time at which opening occurs may be made the basis of the test, which is said to be sensitive to as little as  $0.0004\mu\text{g.}$  of oestradiol propionate or to 0.02 ml. of female blood. Lloyd, Rogers & Williams [1946] have, however, reported that this technique is too variable for precise estimation.

#### ANDROGENS

(i) *Capon comb tests.* As with the oestrogens, large discrepancies occur when different androgens are compared by different test methods. The presence of augmenting substances in extracts interferes with estimates of androgenic activity just as in the assay of oestrogens, but the discrepancies with the androgens are fortunately not so severe when any one test method is considered.

For precisely the same reasons as were discussed when dealing with oestrogens, local application of androgens seems the method of choice. This may be made to the capon comb or to the chick comb. There is no doubt about the suitability of the capon for such work. The bantam capon is easier to prepare and house than capons of heavier breeds, but it is also less sensitive to androgens, and it is rather doubtful whether its use is to be recommended. The suitability of the chick comb test, however, has been very much in question. The relative activity of various androgens when assayed by inunction in oil solution on the comb of the Brown Leghorn capon has been investigated by Dessau [1937] and Emmens [1939]. Both series of results show how much more alike in potency the various androgens are when applied locally, and agree very well. A 20% increase in comb area (Dessau, four daily applications) or a 4 mm. increase in length-plus-height of the comb (Emmens, three daily applications) was considered to be a positive response. A total of about  $2\mu\text{g.}$  of androsterone per bird is sufficient for an assay—which compares very favourably with the 400 or  $500\mu\text{g.}$  needed by intramuscular injection. Apart from its other advantages, the local method enables assays to be made with small urine samples, while a test using intramuscular injection requires more than a 24 hr. sample under average conditions. It has been shown that inunction in alcohol, ether or benzene is, if anything, more efficient than in oil, but the differences are small. The effect of esterification and the presence of impurities have not been properly investigated, but these factors probably do not much affect results obtained by the method of local application.

The inhibitory effect of oestrogens on the response of the capon comb to androgens was investigated by Emmens & Bradshaw [1939]. The results indicate that the ratio of oestrogen to androgen (about 1:1000 by weight) normally found in urine is such that no interference need be anticipated.

A drawback to the inunction technique is that the responses are rather more variable than by injection. Thus, on the average, it is necessary to use about half as many birds again to reach the same degree of inherent precision. It is probable that 17-ketosteroid estimation will hold the field as being as useful an index of endogenous androgen production as is the urinary content of androgens—possibly it is a better index. But a similar calculation to that made for the oestrogens shows that the androgen content of 'neat' urine is of the order needed in direct application to the

comb—a urine with 10 mg. of androgen per litre contains  $1\mu\text{g.}/0.1\text{ ml.}$ , the volume used for each application. Unfortunately, it is probably impossible to apply urine directly to the comb with success, but a simple treatment, perhaps the addition of acetone or alcohol after preliminary concentration of the urine, may render the urine suitable for direct application. Blood might also be amenable to similar treatment.

(ii) *Other tests.* Tests other than the capon comb test are not particularly attractive. They all need either far too much androgen or involve detailed histology which is inappropriate to routine assay. Greene & Burrill [1941] have described a 48 hr. test for androgens using the seminal vesicles of the immature rat which seems to have a reasonably low error and uses about  $50\mu\text{g.}$  of testosterone propionate per animal—this is probably about equivalent to the 400–500  $\mu\text{g.}$  of androsterone needed by injection in the capon. In another paper [Greene & Burrill, 1940] a 24 hr. test using prostatic weight is described. The usual tests employing seminal vesicle or prostate weights in castrated male rats are useless for urine assays, as they require 5 or 10 mg. of androsterone per animal. The mouse, which needs lower weights of hormone, has not been found by Deanesly [1938] to be reliable for such work.

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Dr HASLEWOOD referred to the fact that non-sterol substances are oestrogenic and that there may be a danger that crude extracts of urine give an oestrogenic potency that is not due to sterols.

Dr EMMENS agreed that this might be so in the urine, but was of the opinion that blood assays would not be affected.

**Chemical assay of urinary pregnanediol.** By G. A. D. HASLEWOOD.  
*Guy's Hospital Medical School, London, S.E. 1*

Pregnanediol is said to occur in sterile human urine only in the conjugated form, as the glucuronidate (Bucher & Geschickter, 1941). If this statement is correct, total pregnanediol can be estimated as the glucuronidate or, after hydrolysis, as the free steroid.

Estimation as free steroid was largely developed by Astwood & Jones [1941], by Talbot, Berman, MacLachlan & Wolfe [1941] and, more recently, by MacLagan [1947] and by Marrian & Gough [1946]. The final estimation usually depends on the production of a yellow colour with pregnanediol and  $H_2SO_4$ . This colour is non-specific and the steps leading to the final stage in the method are designed to isolate the pregnanediol in as pure a state and as quantitatively as possible.

Preliminary hydrolysis can be accomplished, more or less successfully, by heating with acid or by enzymic means [Talbot, Ryan & Wolfe, 1943]. The pregnanediol is then extracted with toluene and is partially purified by fractional crystallization from dilute alcohol.

Guterman [1944, 1945] has used this method in a semi-quantitative form as a test for pregnancy.

Sommerville, Gough & Marrian [1947] have recently shown that the rate of cooling during the crystallization of the pregnanediol is an important and hitherto neglected factor in deciding the accuracy of the analysis. The method of estimation of free pregnanediol is at the present time capable of considerable accuracy and specificity, and is sufficiently sensitive for use during the normal female cycle. The value found for male urine is very small ( $< 1$  mg. in 24 hr.).

In Venning's [1937, 1938] original method for estimation of conjugated pregnanediol the fresh or preserved urine was extracted with butanol. Evaporation of the (washed) butanol leaves a residue which may often be made to yield crystals of sodium pregnanediol glucuronide (Na P.G.), which can be weighed. Such a technique is only applicable to urines comparatively rich in Na P.G.; moreover, it has been shown [Mason & Kepler, 1945; Marrian & Gough, 1946] that crystalline Na P.G. may contain glucuronides of pregnane derivatives other than pregnane-3 $\alpha$ , 20 $\alpha$ -diol.

Attempts to make the estimation of Na P.G. more sensitive have involved loss of specificity. Allen & Viergiver [1941] collected the glucuronide on a lead nitrate precipitate, and determined reducing power after hydrolysis. The Tollens naphthoresorcinol reaction has also been used for colorimetric estimation of glucuronide. Isolation of the Na P.G. has been done by methods not involving crystallization, with the result that a considerable fraction of glucuronide definitely not Na P.G. has been included in the estimation.

Bisset, Brooksbank & Haslewood [1947] attempted to evaluate total glucuronide resembling Na P.G., after an entrainment on Ba-phosphate, from butanol extracts of urine. They have called the glucuronide obtained 'pregnanediol-like-glucuronide' (P.L.G.). P.L.G. in the normal female cycle shows a rise in the luteal phase.

Its output in male urine is about 5-10 mg./24 hr. (as Na P.G.), whereas the actual amount of Na P.G. in such urine is probably less than 1 mg./24 hr. [Westphal, 1944]. The chemical nature of P.L.G. is being investigated, and also its concentration in other urines.

Jayle, Crépy & Wolf [1943] and Jayle & Libert [1946] have estimated 'non-phenolic steroid glucuronides' by other quite different methods of isolation (including Venning's acetone crystallization), followed by the application of the naphthoresorcinol reaction. Their figures resemble those obtained by the entrainment method.

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Dr SOMMERVILLE criticized the method described by Dr Haslewood on the grounds of lack of specificity. Of itself, this might not prevent its useful application to certain clinical problems, but they would require careful selection.

**The clinical significance of urinary steroid assays.** By P. M. F. BISHOP.  
*Guy's Hospital Medical School, London, S.E. 1*

## I. NEUTRAL 17-KETOSTEROIDS

It seems to be generally accepted that the neutral (non-phenolic) 17-ketosteroids are excretion products of androgens, of which about two-thirds are contributed in the male by the adrenal cortex and one-third by the testis, and possibly the whole by the adrenal cortex in the female (the ovary being responsible for only a trace). The clinician might therefore expect the estimation of 17-ketosteroids to provide information chiefly about the functional status of the adrenal cortex, and of the testis to some extent. These hopes have been fulfilled in the case of the adrenal cortex to a surprising extent, but before considering the interpretation of the figures in detail, a word of caution is necessary. There is still no agreement about the normal average figure and the normal range of 17-ketosteroid excretion in the human being. Reports on the normal average figure for adult men vary from 9 to 18, and for adult women from 6.4 to 13 mg./day [Callow, Callow, Emmens & Stroud, 1939; Chou & Wang, 1939; Crooke & Callow, 1939; Fraser, Forbes; Albright, Sulkowitch & Reifstein, 1941; Patterson, McPhee & Greenwood, 1942; Scowen & Warren, 1946; Talbot, Butler, MacLachlan & Jones, 1940]. Few figures are available [Fraser *et al.*, 1941; Nathanson, Towne & Aub, 1939] for children. There is, moreover, a considerable degree of overlap of the normal male and female ranges, and it is not possible to identify the sex of the donor of a urinary specimen by estimating its 17-ketosteroid content. Ovariectomized women show figures within the normal female range, as do eunuchs and eunuchoids (about 5 mg./day below the normal male average). With so wide a normal range, 9–28 mg./day in men and 4–23 mg./day in women, according to Scowen & Warren [1946], the clinician is safe in regarding as of serious diagnostic



significance only figures that are either more than twice the normal average or near the zero level.

The highest figures are observed in adrenal cortical tumour and the lowest in Simmonds' Disease, in which the pan-pituitary deficiency affects both adrenals and gonads. Low figures are also found in advanced cases of Addison's Disease in women, but in men suffering from this disease the 17-ketosteroid figure is appreciably higher because the testes remain functional. Between these two extremes there are many clinical conditions in which the figures might be expected to fall outside the normal range.

The 17-ketosteroid figure may be of interest in the differential diagnosis of the following conditions:

(1) *Virilism*. The clinical signs of this condition vary from a mild growth of soft downy hair on the upper lip to marked hypertrichosis, deepening of the voice and an enlarged clitoris. It may be due to an adrenal cortical tumour, a virilizing tumour of the ovary, such as an arrhenoblastoma, pre- or post-pubertal adrenal cortical hyperplasia, compensatory adrenal over-activity due to mild ovarian deficiency, or simply and most commonly to constitutional or racial tendencies. In the first two conditions, both of which are very rare, removal of the tumour is absolutely indicated, and is followed by disappearance of the virilism. Though unilateral adrenalectomy is still performed in cases belonging to any of the other groups, most clinicians are now agreed that this treatment is not followed by remission of the symptoms. It is the duty of the clinician therefore to distinguish between the first two conditions and the rest. In adrenal cortical tumour the 17-ketosteroid excretion is nearly always very high and any figure above 100 mg./day should be regarded as an imperative indication for operative exploration. Callow & Crooke [1944] have, however, reported two cases, one with an adrenal tumour and a 17-ketosteroid figure of not more than 20 mg./day, the other with excretions of 79–100 mg./day and no tumour. The latter may have been a case of pre-pubertal adrenal hyperplasia with which very high figures may be associated. The androgen is excreted chiefly in the form of androsterone, whereas in cases of adrenal cortical tumour large quantities of transdehydroandrosterone are found. 17-Ketosteroid estimations are of no value in the diagnosis of arrhenoblastoma of the ovary, for the figures are within or slightly above the normal range. However, this condition is usually associated with a high degree of masculinization, including an enlarged clitoris, and it is often possible to palpate the ovarian mass. In the remaining groups the 17-ketosteroid figure varies according to the degree of adrenal cortical activity, but once a tumour has been excluded the figure is of academic interest only, for there is no treatment other than depilation or shaving, and operation is not indicated.

(2) *Feminism*. In very rare instances an adrenal cortical tumour occurring in the male has given rise to gynaecomastia. This could, of course, be distinguished from other types of gynaecomastia by the very high 17-ketosteroid output.

(3) *Precocious puberty*. In the male the causes of this condition are interstitial tumour of the testis, adrenal cortical tumour, pineal tumours, tumours in the region of the hypothalamus, and physiological acceleration of the normal rate of growth and development. All but the last are rare. The testicular and adrenal tumours will give rise to very high 17-ketosteroid figures, but can be distinguished from one another

by the presence or absence of an enlarged testicle. The intracranial tumours show normal 17-ketosteroid figures but are often accompanied by signs of involvement of the central nervous system, such as oculomotor disturbances, deafness, or spasticity of the limbs.

(4) *Simmonds' Disease*. In cases of Simmonds' Disease the 17-ketosteroids are usually undetectable. This condition is most frequently confused with anorexia nervosa, in which the 17-ketosteroid figures never reach the zero levels found in Simmonds' Disease although they may be below the normal range on account of the malnutrition. This condition is sometimes misdiagnosed as myxoedema, especially in cases where there has been no loss of weight. Unfortunately the 17-ketosteroid estimation is of little assistance in this differential diagnosis, for very low levels are reported in established cases of hypothyroidism [Friedgood, 1942; Engstrom & Mason, 1944]. Addison's Disease is also often confused with Simmonds' Disease, though the pigmentation in the former, and the characteristic pallor, loss of body hair and low basal metabolic rate in the latter should help to distinguish the two conditions. Here again figures for 17-ketosteroid excretion are not helpful, for in the female case of Addison's Disease they may be at the zero level.

(5) *Hypogonadism and dwarfism*. These conditions may be due to pituitary, thyroid or gonadal failure. Low to very low 17-ketosteroid figures are found in cretinism and pituitary infantilism, whereas primary gonadal failure, whether associated with dwarfism as in Turner's syndrome (ovarian agenesis) or not, as in the case of the male eunuchoid, is characterized by a low normal figure, together with excessive output of F.S.H. Owing to the dearth of figures for normal children, and the low values which they exhibit, observations on 17-ketosteroid excretion are only likely to be of value when the patients are adults.

#### *The use of qualitative 17-ketosteroid determinations*

It has already been mentioned that adrenal cortical tumour may be differentiated from pre-pubertal adrenal cortical hyperplasia because large quantities of transdehydroandrosterone are found in the former condition and of androsterone in the latter. Scowen & Warren [1946] have recently referred to the presence of 11-oxy-corticosteroids in those cases of Cushing's syndrome that are due not to adrenal cortical tumour but to pituitary basophilism. The identification of this cortin-like substance helps considerably in the classification of these two closely allied conditions. Dingemans, Huis in't Veld & de Laat [1946] have recently described a method of fractionating urinary extracts with varying combinations of benzene and ethanol. By this means they have been able to differentiate, with the help of chromatographic-colorimetric analysis, individual 17-ketosteroids present in the extract, and have also confirmed the presence of large quantities of transdehydroandrosterone and of large amounts of another 17-ketosteroid (probably androstenediol-17-one), in the urine from a case of adrenal cortical carcinoma. Little has yet been done in the way of elucidating the complex genetic and endocrine influences in cases of pseudohermaphroditism, but this is certainly a promising line of investigation.

It seems unlikely that much further progress will be made in connexion with quantitative 17-ketosteroid analysis and that qualitative studies will offer greater possibilities of clinical usefulness in the future.

## II. PREGNANEDIOL

At present the chief clinical interest in pregnanediol is its role in the detection of ovulation, its value as a pregnancy test, and its behaviour in certain abnormalities of pregnancy.

(1) *Determination of ovulation.* It is likely that the luteal phase of the cycle can be detected by means of pregnanediol estimations, though this may remain a rather complicated procedure for some time to come, necessitating collection of 24 hr. or pooled 2- or 3-day specimens of urine, possibly on more than one occasion during the cycle. It is therefore well to consider other means for deciding if ovulation has taken place.

The classical method is the endometrial biopsy, which involves a minor operative procedure, carried out sometimes in the out-patient department but preferably in an operating theatre under anaesthesia. A typical secretory endometrium indicates that ovulation occurred in that particular cycle, but anomalous appearances from mixed types of endometria sometimes lead to difficulties in interpretation and do not necessarily indicate a non-ovular cycle.

For the past few years considerable attention has been paid to basal temperature records as a means of detecting ovulation. The temperature is recorded vaginally, rectally or by mouth, by the patient herself, the rectal method probably giving the most reliable records in the 'untrained patient'. My recent experience suggests that oral temperature records may also yield satisfactory results. The waking temperature shows a readily recognizable biphasic pattern, being lower before and higher after ovulation has taken place. It is said that a characteristic dip may occur, indicating the exact day of ovulation, before the temperature records climb up to the 'luteal plateau'. This view may prove to be an unjustified refinement of interpretation, but it is safe to assume that where no biphasic pattern can be traced the cycle is probably non-ovular. Further correlation of temperature charts with endometrial biopsies, laparotomy examination of the ovaries for fresh corpora lutea, and possibly pregnanediol determinations are required before the reliability of this comparatively simple procedure can be gauged.

(2) *As a pregnancy test.* Hain & Robertson [1939], using Venning's method for the extraction of the glucuronidate, noted a high excretion of pregnanediol on the 20th to 24th days of the cycle of a patient already under observation: this change subsequently proved to be due to pregnancy. In the same year Wilson & Randall [1939] suggested that excretion of more than 10 mg./day indicates pregnancy, or that where a period has been missed more than 4 mg./day denotes pregnancy. Buxton [1940] used the estimation in pregnancy diagnosis, and Guterman [1945], employing a qualitative modification of the Astwood-Jones estimation, described a rapid 3 hr. pregnancy test and claimed 93 % accuracy in 222 cases. Reinhart & Barnes [1946], faithfully repeating the Guterman technique, found that the test was only 75 % accurate with 42 % false positives in non-pregnant cases and 16 % false negatives in the pregnant series. Henderson, Maclagan & Wheatley, using a modified quantitative Astwood-Jones technique, have obtained results intermediate between those of Guterman and Reinhart & Barnes. Much seems to depend on the level of pregnanediol excretion chosen as the diagnostic base-line. It seems unlikely that the test as it stands will

achieve the accuracy of the Ascheim-Zondek, Friedman or Xenopus tests, but its simplicity, rapidity and cheapness are commendable.

(3) *Pregnanediol and abortion.* Hain [1940], Hamblen, Cuyler & Baptist [1942], Smith, Smith & Schiller [1941] and others have studied the curve of pregnanediol excretion throughout pregnancy. Before an abortion or a threatened abortion there is often a marked drop in the urinary pregnanediol figure, which since it may precede the clinical signs by some days may prove a useful warning to the obstetrician. In patients with a history of repeated abortions or in whom some abnormality in the course of the pregnancy suggests the possibility of imminent abortion, the closest co-operation between the laboratory and the ante-natal officer may provide an opportunity for successful prophylactic treatment. Nevertheless, the technical difficulties of urine collection, laboratory procedure and co-operation between biochemist and clinician seem to be too great at present to make pregnanediol determinations of general practical value.

### III. OESTROGENS

Up to the present oestrogen estimations have proved of little value to the clinician. For practical purposes they are of value only in the diagnosis of the rare granulosa-cell tumour of the ovary, and, combined with gonadotrophin assay, in determining whether certain cases of primary amenorrhoea are due to primary pituitary or ovarian failure.

The prolonged, intensive and detailed studies by the Smiths and their associates [1941, 1946], of certain aspects of the metabolism of oestrogens and progesterone require mention, since their story of the part played by oestrogens and progesterone in the menstrual cycle and in pregnancy and its toxæmias has aroused considerable interest.

Briefly it is this: by estimation of pregnanediol, and of oestradiol, oestrone and oestriol as well as the total oestrogenic substances recoverable after zinc hydrogenation, at various stages of the menstrual and gestational cycles they claim to have elucidated certain aspects of the metabolism of oestrogen and progesterone. Oestradiol undergoes conversion to oestrone and subsequently to oestriol. This conversion is facilitated by progesterone. If progesterone is withdrawn or deficient, the conversion is retarded, and oxidative destruction of the oestrogens takes place instead, the oestrogen most easily oxidized being oestrone which consequently practically disappears from the urinary samples. The products of oestrogen destruction can be detected by estimating the total oestrogenic potency after zinc-hydrogenation, a process which reconverts them into oestrogen-active material. From the products of destruction the Smiths claim to have isolated a toxin which they believe to be responsible for the disintegration of the menstrual endometrium, to play an important part in parturition and to be one of the chief causes of pre-eclamptic toxæmia. Oxidative destruction of oestrogens not only occurs when progesterone is withdrawn or is deficient, but also when the rate of production of oestradiol diminishes. Thus in order to combat the effects of these destruction products oestrogen and progesterone should be administered. In late toxæmias of pregnancy and eclampsia the Smiths found very high concentrations of serum chorionic gonadotrophins (which they thought to indicate failure of this hormone to be utilized for progesterone production),

and low values of oestrogens and pregnanediol, with relatively high values for oestrogens after recovery by zinc hydrogenation. Actually their values for the individual oestrogens were: oestrone nil, indicating a high degree of oxidative destruction; oestriol low, indicating a low rate of conversion; and oestradiol high, suggesting, not increased production, but a low rate of conversion. The practical application of their theories was to treat cases of pregnancy toxæmia and eclampsia with high doses of oestrogen, progesterone and pregnanediol glucuronidate. Recently they have been using stilboestrol alone. In well-established cases their treatment was only palliative but in early cases the results have been on the whole satisfactory from the curative point of view. Their work has been extended by Priscilla White & Hunt [1940] in the treatment of toxæmia in pregnant diabetics. In a series of thirty-five diabetic women with toxæmia the foetal survival rate was 89 %, whereas in an untreated series of diabetic women with toxæmia it was only 42 %.

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Several speakers referred to the work of Smith & Smith, and it was felt that some of their biochemical methods might be criticized.

In summing up the Meeting the CHAIRMAN first raised the point that research on improving the methods of assay of urinary steroids and their application would be greatly facilitated if adequate amounts of certain pure urinary steroids such as pregnane-3( $\alpha$ ), 20 $\alpha$ -diol and its glucuronide, oestriol and its glucuronide, oestrone sulphate, etc., which are not manufactured commercially, could be made readily available to those requiring them.

It was decided to ask the Secretary to bring this matter up before the Committee of the Society.

The CHAIRMAN welcomed the fact that some active interest is now being taken in this country in the quantitative determination of urinary steroids of adrenal origin other than the neutral 17-ketosteroids. The possible diagnostic value of determinations of such urinary adrenal steroid metabolites had, he thought, been somewhat neglected by British endocrinologists.

He was also most interested to learn from Dr Emmens that under certain conditions it might be possible to obtain oestrogenic responses of equivalent intensity from oestriol, oestrone and oestradiol by intravaginal administration. This raised the further attractive possibility that a reliable bioassay method for total oestrogen in the urine of non-pregnant women might be developed.

Without exception it seemed that the methods for the assay of urinary steroids were not of proved accuracy and reliability. It was worth while to emphasize the deficiencies and limitations of these methods: first to discourage newcomers from using published 'quantitative' methods in an uncritical manner; and second, to encourage one another to develop new and better methods and to improve further the technique of older methods. In the Chairman's opinion, work directed towards improving the methods was far more important and more urgently required than work involving the application of the existing imperfect methods to problems of clinical interest.

Prof. MARRIAN concluded by thanking, on behalf of the Society, Dr Bishop and other members of the staff of Guy's Hospital for their hospitality and for the excellent arrangements which they made for the meeting.

## EIGHTH ORDINARY MEETING

*Held at the Zoological Society, London, on 30 October 1947*

**The clinical value of pregnanediol assays—preliminary report.** By  
G. I. M. SWYER. *Obstetric Unit, University College Hospital, London, W.C. 1*

The Guterman technique for pregnanediol assay has been modified by precipitating the pregnanediol twice during slow cooling (2 hr. in the incubator at 37°) and by using photo-electric colorimetry to give quantitative results. Although the technique is not highly accurate, it is suitable for routine clinical application as a pregnancy test and prognostic aid in threatened abortion.

In twenty-eight known non-pregnant women the highest value obtained was 5 mg./l. and it is unlikely that the assay is sufficiently sensitive to be used for the detection of ovulation.

In pregnant patients on whom about 100 assays have been made, there is considerable variation from day to day, and little significance should be attached to the result of a single assay. Using the criterion of an excretion of more than 6 mg./l. to indicate pregnancy, the results of the pregnanediol assay have correlated very satisfactorily with those of the Friedman test.

Pregnanediol assays have also been carried out in eight cases of threatened abortion. Five cases, in which the pregnancy continued, gave figures of from 16 to 28 mg./l. The remaining three cases, in which the abortion became complete, gave figures of from 0 to 5 mg./l.

**The rapid hyperaemia reaction of the rat ovary to gonadotrophins and its practical application.** By B. ZONDEK and F. SULMAN. *Hebrew University, Jerusalem.*

Hyperaemia as a reaction to gonadotrophins was early described by Zondek, but its value as a pregnancy test was not recognized then because the reaction only appears consistently in a few strains of rats. During the past 3 years well over 3000 tests have been carried out using the hyperaemic reaction. Immature rats have been used because they are more sensitive to chorionic gonadotrophin than mice, and because they more easily withstand the injection of toxic urines. The tests can be carried out within 24 hr. if necessary, and the results can be expressed quantitatively. The amount of gonadotrophic hormone given in a single injection which induces hyperaemia of both ovaries in an infantile rat within 24 hr. has been called 1 hyperaemia unit.

This new test has been compared with existing pregnancy tests as regards its sensitivity to chorionic gonadotrophin, pregnant mares' serum and pituitary gonadotrophin. The results seem to indicate that hyperaemia is evoked mainly by luteinizing hormone and that follicle-stimulating hormone has only an augmenting effect. The test is most sensitive if read 10 hr. after injection, but clinically the 12-24 hr. test has usually been employed. The roughly quantitative method, involving the injection of different quantities of urine, helps in the prognosis of cases of threatened abortion.

**The effect of adrenalectomy on lactation in the rat studied by the paired-feeding technique.** By A. T. COWIE and S. J. FOLLEY. *National Institute for Research in Dairying, Shinfield, Reading*

Experiments using paired-feeding technique have been carried out to see if the reduction in lactational performance after adrenalectomy [Cowie & Folley, 1947a] is due to anorexia. The effect of the substitution of 1 % saline for the drinking water of the adrenalectomized mothers has also been investigated, since such treatment may often overcome anorexia [Ingle, 1944].

The results are given in Table 1.

Table 1. *Mean litter-growth index, and percentage weight change for control and experimental rats*

	No. of rats	Mean litter-growth index/day	Percentage weight-change from day 4 to day 13 of lactation
Experiment no. 1:			
Sham-operated control rats fed <i>ad lib.</i>	8	15.3 ± 0.7	+ 6.9 ± 1.1
Pair-fed sham-operated control rats	15	8.8 ± 0.6	- 16.1 ± 1.8
Adrenalectomized rats	15	6.4 ± 0.6	- 1.1 ± 1.2
Experiment no. 2:			
Pair-fed sham-operated control rats	9	10.4 ± 0.8	- 12.3 ± 2.0
Adrenalectomized rats given 1 % NaCl	9	7.6 ± 0.8	+ 5.1 ± 1.7

In both experiments the mean litter-growth index [Cowie & Folley, 1947b] of the adrenalectomized rats (with or without additional saline) was significantly lower ( $P < 0.01$ ) than that of the sham-operated pair-fed controls. Part of the reduction in the lactational performance following adrenalectomy, therefore, is not due to anorexia, and cannot be overcome by administration of NaCl.

Pair-fed control rats were very much more active than the adrenalectomized animals, and lost much more weight. The interpretation of the results of similar experiments that involve the equalized food intake technique may be more difficult than appears at first sight owing to these changes in body weight, and the excessive activity resulting from hunger.

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**Assay of corticotrophic hormone in body fluids.** By D. S. COOKE, E. GRAETZER and M. REISS. *Endocrinological Research Institute, Bristol Mental Hospitals*

Sayers & Sayers' method of corticotrophic hormone assay, which is based on the decrease in ascorbic acid content of the adrenals, is thirty to seventy-five times more sensitive than the assay method based on the disappearance of the sudanophobic zone in the adrenals of hypophysectomized animals.

When testing body fluids the method should be carried out only on hypophysectomized rats. Infantile rats are unsuitable since the ascorbic acid content of the gland is reduced 50% by as little as 1 ml. of unconcentrated urine, which mobilizes endogenous corticotrophic hormone.

The corticotrophic hormone in urine is concentrated by passing the urine (pH 3.2) through a permutit column and eluting this column with 35% alcohol containing 10% ammonium acetate. The hormone is then precipitated from the eluate by increasing the alcohol concentration to 85%. After washing with acetone and ether, and drying *in vacuo*, between 15 and 25 mg. of precipitate per litre remain. This precipitate can be dissolved in distilled water and a quantity equivalent to 250 ml. of urine from normal individuals, injected into hypophysectomized rats, is usually sufficient to decrease the ascorbic acid content by 50–80 mg./100 g. of fresh adrenal.

To extract the corticotrophic hormone in plasma, inert proteins are first precipitated and removed at 50% acetone concentration. The acetone concentration of the filtrate is then raised to 85%, and the resulting precipitate washed with acetone and ether, and dried. If this precipitate, corresponding to 5–10 ml. of original plasma, is dissolved in distilled water and injected intravenously to hypophysectomized rats, there is a decrease of 50–100 mg. of ascorbic acid/100 g. of fresh adrenal tissue.

**The effect of sex hormones on the sebaceous glands of the female albino rat.**

By F. J. EBLING. *Department of Zoology, University of Bristol*

A technique was described for estimating the degree of development of the sebaceous glands in the rat. Oestradiol benzoate in large doses (1000 i.b.u./day for 36 days) caused atrophy of the sebaceous glands in the female rat. Moderate doses (10 i.b.u./day)



had a less-marked effect, but still decreased the total amount of gland material. Testosterone propionate increased the size and activity of the glands. Treatment with progesterone had no effect.

**The Mittelschmerz.** By P. L. KROHN. *Department of Anatomy,  
The Medical School, Birmingham, 15*

The cyclical occurrence of inter-menstrual pain (Mittelschmerz) in a woman aged 27 has been followed for a period of about  $3\frac{1}{2}$  years. The pain is quite characteristic and easily recognized. It begins suddenly in one or the other iliac fossa and gradually becomes merged into a generalized pelvic pain which lasts for up to 24 hr. The pain did not occur before the age of about 16, and its incidence was irregular for several years after that.

The onset of the pain in relation to the menstrual cycle has been extremely regular, the mean interval from the pain to the beginning of menstruation being  $13 \pm 0.2$  days and from menstruation to the onset of the pain  $17 \pm 0.4$  days.

The pain was absent throughout pregnancy and lactation; about 3 weeks after the end of lactation it was again recorded, to be followed 10 days later by the return of menstruation.

The pain has occurred nineteen times on the right side and twelve times on the left, but never twice in a single cycle. There does not appear to be any regularity or alternation in the side which is chosen.

Correlation of the incidence of the Mittelschmerz with the waking oral temperature during two cycles shows that the pain begins during the rise from the low pre-ovulatory phase temperature to the higher figure usually thought to indicate ovulation and the beginning of the secretory phase.

**The influence of thyroglobulin and iodinated casein on the respiration of rat liver slices.** By H. G. WILLIAMS-ASHMAN. *Department of Biochemistry,  
University College, London*

The immediate effects of thyroglobulin and iodinated casein on the respiration of adult male rat liver slices have been studied by the Warburg method.

Thyroglobulin was prepared from sheep's thyroids by a modification of the Oswald method suggested by Rossiter [1940]. The iodinated casein was a Boots commercial preparation, purified by dialysis and re-precipitation. The results obtained are summarized in the following table.

No. of exps.	Substance added	Range of concentrations added (mg.)	Mean oxygen consumption in $\mu$ l. oxygen consumed/30 min./g. fresh tissue		Percentage change compared with control period
			Control period	Experimental period	
11	Thyroglobulin	2-10	618	631	+2
	Controls	—	578	598	+3
8	Iodinated casein	6-10	631	620	-2
	Controls	—	594	597	0

It is concluded that neither thyroglobulin nor iodinated casein in the concentrations used have any significant immediate effect on the respiration of rat liver slices. These results do not agree with those of Canzanelli & Rapport [1937] who, using a slightly different technique, reported an increase in the oxygen consumption of rat liver slices to which thyroglobulin had been added *in vitro*.

#### REFERENCES

- Rossiter, R. J. [1940]. *J. Endocrinol.* **2**, 165.  
Canzanelli, A. & Rapport, D. [1937]. *Endocrinology*, **21**, 779.

**The prolonged administration of oestrogen or androgen to immature female monkeys.** By P. L. KROHN. *Department of Anatomy, The Medical School, Birmingham 15*

One immature female rhesus monkey was given 1 mg. oestradiol dipropionate daily, and another 200 mg. testosterone propionate weekly (in 4 daily injections) for 899 days. The animals were about 12 months old at the start of the experiments.

The main findings are reported below.

*General growth and development.* At the end of the experiment the oestrogen-treated animal weighed only half as much as the control monkey, and its dental development had been greatly retarded. The weight of the androgen-treated monkey differed only slightly from the control, but growth of the hair, the muscles of the neck and the clitoris had all been stimulated.

*Pituitary.* It is usually considered that one of the results of prolonged oestrogen treatment is an enlarged pituitary, and the formation of a chromophobe adenoma. However, the oestrogen-treated pituitary was smaller, while the androgen-treated gland was larger, than normal. In both monkeys there had been an increase in the proportion of acidophils at the expense of the other cell types. (Acidophils 60-65 %, chromophobes 30-35 %, erythrosin-staining basophils not more than 5 %.)

*Ovaries.* The appearance of the ovaries could not be ascribed to indirect effects of either hormone through the pituitary. In the case of the oestrogen-treated animal the small ovaries contained only a few normal follicles with antra, the rest being made up of small atretic follicles consisting of a dying ovum surrounded by one or two layers of hyalinizing granulosa cells. The appearance of the ovary did not resemble the condition found after hypophysectomy.

Androgen treatment induced the formation of numerous small healthy follicles without the production of any corpora lutea. These observations confirm others that have already been made in monkeys (Green & Zuckerman [1947]).

*Reproductive tract.* The oestrogen treatment induced squamous metaplasia throughout the cervix and in the lower part of the uterus. Much of the uterine endometrium had been replaced by a nodular overgrowth of the fibrous stroma; that which remained showed signs of cystic hyperplasia. There was only one short period of menstrual bleeding during the course of the experiment.

The androgen-treated uterus contained a number of slightly cystic straight glands, and there were no signs of progestational changes. The epithelium lining the cervix

was high columnar and was actively secreting mucus. The vaginal mucosa was thin, inactive and not keratinized. Menstrual bleeding did not occur at any time.

Structures similar to seminal vesicles and Cowper's gland were found at the sides of the cervix and vagina respectively.

*Mammary glands.* Both hormone treatments induced similar changes in the mammary glands, which were made up of tightly packed lobules of alveoli, but the androgen-treated glands were about 50 % larger.

*Cancerous changes.* No evidence of malignant growth was seen in any of the tissues that were examined.

#### REFERENCES

Green, S. H. & Zuckerman, S. [1947]. *J. Endocrinol.* (In the Press.)

